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THE SODIUM CHANNELS OF MAMMALIAN SKELETAL MUSCLE

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE SODIUM CHANNELS OF MAMMALIAN SKELETAL MUSCLE submitted by Donald Cheung in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

TTX is a very specific inhibitor of the Na conductance in many excitable membranes. Mammalian skeletal muscles, which are normally susceptible to 10^{-6}M TTX, become partially resistant after denervation. It was thus of interest to study and compare the Na channel system of the mammalian skeletal muscle before and after denervation. Initially ^{3}H-TTX binding studies were done on the normal and denervated muscles. However, the data were not precise enough to allow unequivocal interpretation.

Electrophysiological experiments yielded some interesting results. The maximal rate of rise of the action potential underwent a temperature transition at around 32°C, so that essentially there were two temperature components - at low temperature, the Q_{10} was 1.07, and at normal physiological temperature, the Q_{10} was 1.29. The TTX sensitivity also changed with temperature. At low temperature, the TTX sensitive range was about 10^{-8} - 10^{-6}M while at 35°C, the range was 10^{-9} - 10^{-6}M. Imidazole could reduce the maximal rate of rise at 35°C to the level as that of low temperature and produced no obvious effect at 25°and 30°C. The TTX sensitive range at 35°C in the presence of imidazole was also about 10^{-8} - 10^{-6}M. The maximal rate of rise of the action potential was reduced at all temperatures after denervation.

The binding parameters and the electrophysiological observations were interpreted in terms of several possible models. There was no conclusive evidence to support the validity of any of the models, however, the two component system, which requires two types of Na chan-

nels in both the normal and the denervated muscles, appears to be more easily interpretable. In this model, the electrophysiological observations were also more compatible with the empirical binding parameters.

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INTRODUCTION

The enhanced movement of ions during an action potential has been described by Hodgkin and Huxley in terms of two major permeability changes, an early one for sodium, and a delayed one for potassium (Hodgkin and Huxley 1952a, 1952b). The early change is due to a transient inward flow of Na ions, moving through what have come to be known as the Na channels. The characteristics of the Na channels could be described by two gating factors - m for Na activation and h for Na inactivation. In the squid giant axon, the Na current is proportional to m^3h.

The delayed permeability change is the result of a maintained outward flow of K ions. The potassium channels have only one gate, n, for K activation. In the squid giant axon, the K current is proportional to n^4. These gating factors of the Na and the K channels are functions of time and membrane potential. The success of interpretating many of the excitation processes in terms of two major ionic components makes the Hodgkin-Huxley model extremely attractive.

The Hodgkin-Huxley equations suggest that axons would be inexcitable in the absence of Na ions, this was borne out experimentally although other cations, such as Li and ammonium, could substitute for Na (Tasaki et al 1966). External Rb ion is a K substitute with a permeability almost equal to P_K (Muller-Mohnssen and Balk 1966). The idea that there are two different discrete, localized pathways for Na and K is best demonstrated by pharmacological studies of squid giant axons under voltage clamp conditions.

When the squid giant axon is internally perfused with 40mM

TEA, the potassium current is eliminated leaving a current that behaves as the sodium current described by the Hodgkin-Huxley equation (Armstrong and Binstock 1965). The ability of TEA to block the K channels is believed to lie in the fact that the ethyl groups around the charged nitrogen gives this portion of the molecule a diameter of about 8 Å, which is approximately the size of a K ion with a complete hydration shell (Armstrong 1975). Armstrong and Binstock (1965) suggested that TEA is swept into the membrane with the outward flux of K ions, blocking the channels from further movement of K.

Tetrodotoxin (TTX), which will be discussed more fully later, eliminates the sodium current leaving a current that resembles the potassium current of the Hodgkin-Huxley model. The experiments with TEA and TTX gives strong evidence for the existence of separate Na and K channels. Further support of this hypothesis comes from internal perfusion experiments with the proteolytic enzyme pronase. After pronase treatment, the sodium current activates in the usual way, however, it does not inactivate. The onset or the amplitude of the potassium current is not affected (Armstrong et al 1973), but could be independently blocked with TEA.

It is thought that ions traverse excitable membranes during an action potential by means of pores rather than carriers. This is because the excitable membrane can transport ions much more rapidly than any known membrane carriers. Estimates of K channel conductance are at least two orders of magnitude higher than any known carriers (Lauger 1972). A major reason for slow movement of an ion carrier is electrostatic. According to calculations by Parsegian (1969), an ion passing through a lipid membrane in a carrier faces an energy barrier

of at least 16kcal/mole, corresponding to a Q_{10} of at least 2.4. The Q_{10} of sodium permeability was found to be 1.3 and that of potassium 1.2 in the myelinated nerves of Xenopus (Frankenhauser and Moore 1963), which would correspond to an energy barrier of no greater than 3kcal/mole. This is much too low for a carrier, but not far from Parsegian's estimation for a pore.

THE SODIUM CHANNEL

Based on permeability ratios estimated from the constant field equation of organic and metallic cations, Hille developed a rather specific geometric model for the portion of the Na channel that is ion selective (Hille 1971, 1972, 1975). The model calls for a rectangular hole of about 3 x 5 Å as all the permeant ions can fit through an aperture this size. Hille (1972) also postulated that the constriction of the pore would be lined with oxygen atoms. Experimentally, it was observed by Hille (1972) that although hydroxylammonium ion and methylammonium ion are similar in size and dimension, only the former can pass through the Na channel. This was argued by Hille as evidence that the pore's constriction is lined with oxygen atoms as the hydroxyl hydrogen of hydroxylammonium ion can form a hydrogen bond with the oxygen of the pore, with the result that the hydroxyl group and the entire ion can pass through the pore. The hydrogens of the methyl group of methylammonium, however, cannot form hydrogen bonds, rendering it too large (3.8 Å in diameter) to go through.

Hille also proposed that the Na channel contains a high field strength anion because titration with acid produces a block of Na permeability which is half complete at pH 5.2 (Hille 1968, Wood-

hull 1973). This block follows the titration curve of an acidic group, the most likely candidate being carboxylic acid. Because of the small size of the orifice, the permeant ions have to be dehydrated and stabilized by the strong negative charge. The selective filter, which discriminates the permeability of different ions, would be the highest energy barrier and lies just behind this negative charge. Most cations can bind to this negative charge, however, only the permeant ions can get through the short narrow selectivity filter while still remain coordinated with the "catalytic" acid group. This is the rate limiting "activated complex". TTX, a potent blocker of the Na current, presumably acts by binding with this acid group, thus preventing Na from entering the pore (Henderson et al 1974).

An efficient pore mechanism should combine selectivity with high transport rates (Lauger 1973). The high selectivity of the Na channel and a rate of transport in the order of 10^8 to 10^9 Na ions per second make the model proposed by Hille even more attractive.

In order to delineate the structural properties of the Na channel, biochemical and enzymatic reagents have been employed to see how they affect the excitation process. Heavy metal ions such as Hg, Ag, Cu and the more selective SH-reactive agents such as N-ethylmaleimide (NEM) and p-chloromercurobenzoate (PCMB) all reduce the excitability of axons in proportion to their ability to form insoluble salts with sulfides. Their action could be prevented or reversed with cysteine or glutathione (Castillo-Nicolau and Hufschmidt 1951, Huneens-Cox et al 1966, Takahashi et al 1958). These findings suggest the presence of essential SH groups closely associated with

the Na channel. This in turn implies that the channel is composed at least in part of protein.

However, application of a wide variety of proteases externally to the squid and lobster axons were found to have no effect on the resting and action potentials. The enzymes tried include trypsin, α-chymotrypsin, γ-chymotrypsin, papain, ficin, carboxypeptidase A and carboxypeptidase B (Narahashi and Tobias 1964, Nelson 1958, Sevcik and Narahashi 1973, Tasaki and Takenaka 1964, Tobias 1955, 1958, 1960). When internally applied, these enzymes do hinder the conductance mechanism. Tritiated-TTX binding experiments also confirm such findings. Only two proteases, chymotrypsin and pronase, were found to be effective in reducing TTX binding with garfish olfactory nerve (Benzer and Raftery 1972).

The involvement of membrane phospholipids in excitation has also been studied using phospholipases, which hydrolyze phosphoglycerides at very specific sites. Phospholipase A depolarizes the lobster axon and blocks conduction (Narahashi and Tobias 1964). Voltage clamp analyses with internally perfused squid axons show that this enzyme suppresses transient current and the steady state current, and the leakage current is affected only after prolonged perfusion (Abbot et al 1972). Phospholipase C, when perfused internally through the squid axon, also causes a gradual depolarization followed by conduction block (Freeman 1969). However, for squid giant axons, externally applied phospholipases exert no effect at all.

Treatment of garfish olfactory nerve homogenates with phospholipase A inhibits binding of TTX while phospholipases C and D are not effective. Prior treatment of phospholipase A followed by

application of proteolytic enzymes results in greater reduction of binding (Benzer and Raftery 1972). Similar studies on axolemma preparation from lobster walking leg nerves also show that while phospholipase A and phospholipase C reduce TTX binding to various degrees, proteases such as pronase, trypsin and chymotrypsin have no direct influence on the binding properties of TTX (Villegas et al 1973).

These results indicate that the protein part of the channel is probably embedded in a phospholipid environment, which then may explain its immunity against direct attack of proteolytic enzymes. Recent TTX binding studies with subcellular fractions from lobster walking leg nerve (Barnola et al 1973) and from garfish olfactory nerve (Villegas et al 1974) show that the channels are located on the plasma membrane. Unfortunately, morphological studies using electronmicroscopic techniques to identify such entities as Na or K channels have so far been unsuccessful.

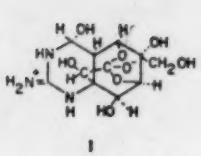
However, the success in electrophysiological measurements using the Hodgkin-Huxley equations and permeability measurements from voltage clamp experiments do indicate very convincingly the existence of such specific channels. The real challenge then is to characterize and isolate the channels so that not only the physical properties of these channels could be studied, but also the basic mechanism of membrane excitation could be unravelled.

TETRODOTOXIN (TTX)

TTX, the non-protein extract from the ovaries and livers of the pufferfish, was first reported to have an inhibitory effect on the action potential of frog skeletal muscle in 1960 (Narahashi et al 1960). This action was found to be highly specific as it selectively blocks the membrane transient conductance increase upon depolarizing stimulation. This unique property of TTX was demonstrated more conclusively with the squid giant axons (Nakamura et al 1965), lobster giant axons (Narahashi et al 1964), and the frog nodes of Ranvier (Hille 1968) under voltage clamp conditions. Only the Na current was blocked by TTX while the K and leakage currents were not affected.

The action of TTX is to block the ionic channel through which the transient current flows. For most excitable membranes, this current is carried essentially by Na ions alone, although TTX could block this current regardless of what ion species is serving as the Na substitute (Moore et al 1967, Rojas and Atwater 1967).

TTX is a very complex molecule. It is assumed that the guanidinium group is essential for its activity. This is substantiated by the fact that saxitoxin (STX), also a non-protein poison that comes from the dinoflagellate Gonyaulax catanella and having identical effect on the sodium conductance as TTX, also contains the guanidinium group (Wong et al 1971). It is also known that guanidine itself could substitute for sodium and penetrate the transient channel to produce action potentials (Watanabe 1967).(Fig. 1)



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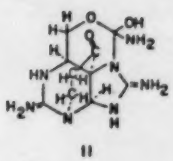

II

Fig. 1. Structure of TTX(I) and STX(II).

Internal perfusion studies using the squid giant axon offer an excellent opportunity to study the site where TTX acts. Twenty-six minutes of internal perfusion with up to 10^{-6}M TTX do not in any way hamper the sodium conductance whereas only 10^{-7}M is required to block the conductance completely from the outside (Narahashi et al 1966, 1967) From measurements of the dose-response curves under voltage clamped conditions with different nerve and axon preparations, and also from tritiated TTX binding studies, it was concluded that TTX is bound to its receptor on a one-to-one basis (Cuervo and Adelman 1970, Hille 1970, Colquhoun et al 1972).

Recent binding experiments have shown that TTX acts at a metal cation binding site (Henderson et al 1974). It was suggested that this site is the principal coordination site for cations, normally Na, as they pass through the membrane during an action potential. Both these physiological and binding observations fit very well into the pore model of the Na channel as proposed by Hille (Hille 1971, 1975). As the TTX molecule contains several hydroxyl and ammonium groups which could form hydrogen bonds with the oxygens lining the pore, the positively charged TTX is bound to the negatively charged acid group within the channel.(Fig. 2)

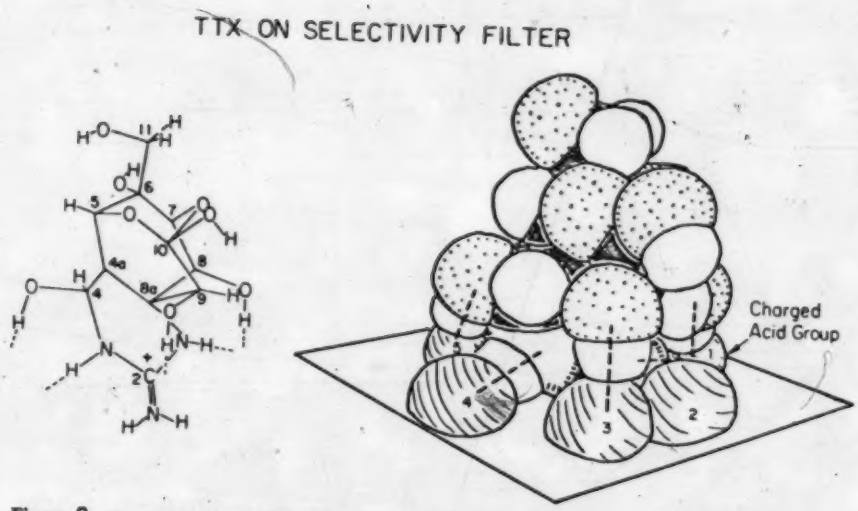

Figure 2 A perspective view of the molecular structure of tetrodotoxin and a space-filling model showing the hypothesis for association with oxygens of the selectivity filter. The two drawings have exactly the same orientation and "magnification." Oxygens of the filter are numbered (1–6) as in Hille and shaded with curved lines. TTX model: Carbons black, oxygens dotted, nitrogens hash-lined, hydrogens all white, hydrogen bonds dashed. The drawing is not meant to imply that oxygens of the selectivity filter are the only components of the TTX receptor. (from Hille 1975)

TTX BINDING STUDIES

As demonstrated in electrophysiological investigations, the high specificity of TTX action makes it very attractive for biochemical use in the labelling, characterization, and isolation of the Na channel if indeed the TTX molecule does act by binding directly to the Na channel as proposed by Hille. In such characterization and isolation studies, especially with tissue homogenates when no direct physiological measurements could be taken simultaneously as control, it is necessary that the observed TTX-membrane interaction is relevant to that under normal physiological conditions. In other words, the range

of toxin concentration used must be close to that which is active in electrophysiological studies. From the many publications of such binding and characterization studies of TTX on various nerve preparations, it is quite evident that TTX does seem to be an adequate tool in many respects.

All the binding experiments carried out so far make use of tritiated TTX of high specific activity. Such labeling could be achieved by a modified Wilzbach exchange method (Dorfman and Wilzbach 1959). Binding studies have also been carried out before labeled TTX became available by bioassay methods although the scope of applicability in such cases are rather limited. By such a method, the number of binding sites of the lobster walking leg nerve had been estimated. (Moore et al 1967).

More accurate estimation of the number of TTX binding sites has been achieved using radioactive binding techniques. As is evident from Table 1, the binding estimation and the dissociation constants determined agree very well with experiments done with bioassay and physiological techniques. The binding component shows saturation at the physiologically active concentration range of TTX.

Binding of TTX is competitively inhibited by STX (Colquhoun et al 1972, Henderson and Wang 1972). Lidocaine and batrachotoxin, both thought to have a direct effect on the Na channels by acting internally, have no obvious effect on TTX binding (Colquhoun et al 1972). This agrees well with the internal perfusion studies of the squid giant axon which show that TTX could act only on the outside of the membrane

Table 1. Binding characteristics of TTX in excitable membranes as determined by various techniques

TISSUE PREPARATION	# OF SITES/um^2	K_D (nM)	METHOD	REFERENCE
Garfish Olfactory Nerves	3.9	8.3	Binding	Benzer & Raftery 1972
	(purified G_{Na}^2 fraction)	5.5	Binding	Chacko et al 1974
	2.5	10.1	Binding	Colquhoun et al 1972
	-	6.0	Binding	Henderson & Wang 1972
	-	12.0	^{22}Na flux	Henderson & Strichartz 1974
Nodes of Ranvier - frog	-	3.6	Voltage Clamp	Schwartz et al 1973
xenopus	-	3.4	Voltage Clamp	
Rabbit Brain Nerve Endings	22	25	Binding	Hafeman 1972
Lobster Walking Leg Nerve	13	-	Bioassay	Moore et al 1967
	16	10	Binding	Colquhoun et al 1972
Mouse Neuroblastoma C 1300	-	11	^{22}Na uptake	Catterall & Nirenberg 1973
Squid Giant Axon	-	3.31	Voltage Clamp	Cuervos & Adelman 1970
Rabbit Vagus Nerve	27	3.0	Binding	Colquhoun et al 1972

(Narahashi et al 1966). Divalent cations such as Ca, Mg, Ba and Sr, trivalent ions such as La and Sm as well as monovalent cations such as Li, Tl and H compete reversibly with TTX (Henderson et al 1974). The competition confirms the existence of a strong negatively charged group which is thought of as the Na coordination site during an action potential. Binding studies done in subcellular fractions pinpoint the localization of the Na channels as on the plasma membranes (Villegas et al 1973, Barnola et al 1972).

Preliminary characterization studies have shown that the Na channel is at least in part composed of protein which is embedded in a phospholipid environment (Benzer and Raftery 1972) Solubilization of garfish olfactory nerve extracts yields a soluble TTX binding component of 500,000 molecular weight (Henderson and Wang 1972). Despite the apparent success in corroborating the binding data with that of the physiological observations, further attempts in purifying and isolating the TTX binding sites have not been very fruitful.

As pointed out by Narahashi (1974), there are several technical difficulties in isolating the TTX receptor. The intrinsic problem is of course the scarcity of the transient channels. Therefore, an extremely sensitive method for detecting the binding and a high degree of purification of both the labeled TTX and membrane preparations are required. Another problem is also intrinsic in that the binding component is very labile so that isolation or purification of the component may leads to alteration of its property.

The best example illustrating the difficulty is the work by Henderson and Wang (1972). They were able to solubilize and retain TTX binding activity from the garfish olfactory nerves only with the

mildest treatment with Triton X-100 and sodium cholate. Other detergents such as sodium deoxycholate, Tween-80, digitonin and sodium dodecyl sulfate failed to solubilize the binding component. Further purification of the solubilized fraction led to loss of activity. If the solubilized material was kept at 0 - 4°C, very little drop in binding activity was observed over a period of several days. However, even after one hour at room temperature, the binding activity would completely disappear. The half-life was about 30 mins at 20°C. This is in contrast to its stability while still in the membrane.

In addition to the intrinsic difficulties discussed above, there are other limitations to the binding method. Because of the scarcity of the binding sites, it is desirable to have the label on TTX as hot as possible. However, the only available label at the moment is tritium. Introduction of other stronger radioactive labels poses a great technical problem as any modification of the TTX molecule greatly reduces its activity (Deguchi 1966) and attempts to synthesize the molecule chemically have so far been unsuccessful. Despite its high specificity in action, TTX also binds non-specifically to the membrane (Colquhoun et al 1972, 1974, Almers and Levinson 1975). Therefore, it is necessary to differentiate the true binding of TTX to the Na channels and the non-specific binding.

Although there are limitations to the method, the merits and validity of such undertakings should not be disregarded. Obviously further refinement of the technique, in particular a more powerful radioactive label and improvement in membrane subfractionation or solubilization procedures, would allow the full potential of this approach to be achieved. At this point in time when there is no

better means to allow for the exploration of the nature of the Na channel, and if we are fully aware of the limitations of the method, TTX binding studies prove to be a sound approach to the problem.

THE NATURE AND OBJECTIVES OF PROJECT

The effect of TTX on skeletal muscle was first reported by Narahashi et al in 1960 of the sartorius muscle of the frog. It was found that 10^{-7}M TTX could eliminate action potential generation although the resting membrane potential and the resting membrane resistance underwent little or no change.

Unfortunately, subsequent studies of TTX action were concentrated on nerve prepartions, for the obvious reason that the voltage clamp technique is well suited to that kind of study. It was not until very recently that moderate success has been achieved in voltage-clamping the skeletal muscle fiber. The renewed interest of the action of TTX on skeletal muscle began in 1970 with the works from Thesleff's laboratory. One very interesting discovery they made was that while the action potential of rat skeletal muscle could be abolished with 10^{-6}M TTX, concentrations of up to 10^{-5}M could not block the action potential completely after surgical denervation (Redfern et al 1970). This finding immediately raises two questions:

1. IS THE TTX RECEPTOR (Na CHANNEL) IN SKELETAL MUSCLE THE SAME AS THAT IN NERVE?

Of all the TTX sensitive nerve preparations studied, 10^{-7}M TTX was sufficient to abolish the regenerative process. These include squid giant axon (Nakamura et al 1965), lobster giant axon (Narahashi et al 1964), frog nodes of Ranvier (Hille 1968a, 1968b), rabbit vagus nerve (Keynes et al 1970, Colquhoun and Ritchie 1972), frog

sciatic nerve (Strong et al 1973), and the nerve axons of the cauda equina of the rat (Evans 1969).

For the mammalian skeletal muscles, the effective range of TTX is much broader, stretching from 10^{-9}M to 10^{-6}M. The muscles studied so far are the extensor digitorum longus (EDL) of the rat (Redfern et al 1970), the soleus muscle of the rat (Albuquerque and Warnick 1972), and the extensor digitorum longus of the mouse (Grampp et al 1972).

As was reflected by the effective range of TTX concentration, there is no reason to believe that the TTX receptor should be the same in skeletal muscles as those in nerves. Indeed, one should not even expect the Na channel to be exactly the same from one nerve preparation to another. In fact, the regenerative mechanism of the skeletal muscle is quite different from the nerve. Although the action potential of skeletal muscle is also the consequence of a rapid Na permeability change (Nastuk and Hodgkin 1952, Adrian et al 1966, 1968, Idelfonse and Roy 1972), in general the spike in muscle is slower in development than in nerve and its velocity of propagation is about one-tenth (Gopfert and Schaefer 1938). Skeletal muscle also exhibits a negative afterpotential whereas the afterpotential is generally positive in nerve.

From the above discussion, it is evident that the TTX receptor of skeletal muscle may not be exactly the same as those in the nerve, although one would expect them to share many properties, both physiologically and structurally.

2. HOW DOES MAMMALIAN SKELETAL MUSCLE DEVELOP TTX RESISTANCE AFTER DENERVATION?

Following denervation, the membrane properties of skeletal muscle changes markedly. Morphological studies indicate that while the muscle fibers decrease in diameter, there is an overdevelopment of the sarcoplasmic reticulum after denervation (Margreth et al 1972). Accompanying these changes is an increase in protein synthesis (Muscatello et al 1965).

Electrical measurements also show an increase in membrane capacitance, time constant and membrane resistance of a unit area (Albuquerque and McIsaac 1970, Albuquerque and Thesleff 1968). At the same time, the resting membrane potential decreases from about 72mv in the normal innervated rat extensor digitorum longus to about 55mv.(Table 2)

TABLE 2

ELECTRICAL MEMBRANE CONSTANTS OF THE INNERVATED AND DENERVATED EXTENSOR MUSCLES

Days	RMP (mv)	R_m (Mohm)	λ (mm)	τ_m (msec)	ρ (calc) (Ω)	ρ (exp)[2] (Ω)	Rm (ohm cm²)	Cm (μF/cm²)
Control	77 ± 1.2 (50)	0.41[1] ± 0.05	0.53 ± 0.07	1.5 ± 0.2	19.6 ± 1.3	18.8 ± 1.9	515 ± 79	2.8 ± 0.5
1	70 ± 3.9 (9)	0.48 ± 0.03	0.52 ± 0.1	1.3 ± 0.2	18.5 ± 1.2	16.0 ± 0.8	525 ± 95	2.5 ± 0.7
3	57 ± 2.6 (9)	0.40 ± 0.82	0.60 ± 0.1	2.0 ± 0.3	21.1 ± 0.8	17.0 ± 0.7	580 ± 44	3.4 ± 0.6
4	64 ± 3.9 (17)	0.59 ± 0.1	0.59 ± 0.2	2.1 ± 0.4	17.0 ± 1.1	16.0 ± 2.3	703 ± 61	3.0 ± 0.8
5	62 ± 3.7 (19)	0.65 ± 0.2	0.53 ± 0.1	2.6 ± 0.5	16.0 ± 1.7	15.0 ± 1.6	668 ± 140	3.9 ± 1.8
7	59 ± 5.2 (9)	0.67 ± 0.3	0.70 ± 0.2	5.1 ± 0.5	17.0 ± 2.3	14.3 ± 2.1	960 ± 109	5.3 ± 0.6
10	59 ± 2.4 (11)	0.68 ± 0.2	0.68 ± 0.2	4.5 ± 0.5	16.3 ± 1.1	13.1 ± 1.6	980 ± 89	5.6 ± 1.5
13	54 ± 4.1 (10)	0.82 ± 0.3	0.70 ± 0.1	6.4 ± 0.8	15.7 ± 0.8	10.1 ± 1.4	1250 ± 130	5.1 ± 1.2
19	50 ± 2.1 (9)	0.91 ± 0.1	0.67 ± 0.1	6.9 ± 1.7	12.0 ± 1.0	8.0 ± 0.9	1310 ± 110	5.3 ± 1.8
25	53 ± 2.7 (8)	0.87 ± 0.1	0.71 ± 0.09	6.0 ± 0.5	15.3 ± 1.0	9.1 ± 1.1	1185 ± 146	5.1 ± 2.0
45	53 ± 2.9 (8)	0.98 ± 0.1	0.79 ± 0.1	7.5 ± 1.0	13.7 ± 2.1	8.3 ± 1.3	1610 ± 103	4.7 ± 0.8

(from Albuquerque & Thesleff 1968)

In both the rat extensor digitorum longus and soleus, the threshold for excitation increases after denervation. The amplitude and the rate of rise of the action potential also decrease (Albuquerque and Thesleff 1968). TTX at 10^{-6}M can abolish the action potential of the rat EDL, whereas up to 10^{-5}M TTX cannot completely eliminate the spike mechanism following denervation (Redfern and Thesleff 1971b, Albuquerque and Warnick 1972). This regenerative response that is resistant to TTX is also Na dependent (Redfern and Thesleff 1971b, Colquhoun et al 1974).

With the technique of anodal polarization, no correlation was found between resting membrane potential and the maximal rate of rise of the action potential in the depolarized fibers (Redfern and Thesleff 1971b). Therefore it is unlikely that the fall in rate of rise following denervation is secondary to the reduction in resting membrane potential. Denervation thus produces a genuine reduction in the rate of rise of the action potential in the muscle fiber. The change in the time constant following denervation, which might affect the maximum rate of rise, however, is not coincidental with the fall of the rise rate (Albuquerque and Thesleff 1968).

The reduction in the amplitude and the maximal rate of rise of the action potential following denervation could be explained by a reduction of the number or the efficiency of the membrane sites responsible for the action potential. This still would not explain the phenomenon of the TTX resistant action potential. Therefore, qualitative changes at these membrane sites must occur. It is possible that upon denervation, the deprivation of certain neurotrophic factors may alter the gene expression resulting in a structural/conformational change

of the membrane sites (Guth et al 1970, Samaha et al 1970). This idea is supported by the finding that inhibitors of protein synthesis such as actinomycin D inhibit the development of extrajunctional cholinergic receptors, TTX resistant action potential, and the fall in resting membrane potential of the denervated mouse EDL (Grampp et al 1972).

The aim of this research project then is to try to answer the questions raised above. The use of TTX binding seems to be a reasonable approach to the problem as the method, despite its limitations, have been well proven in studies with the nerve preparations. The attempt in this project is not to go so far as to isolate the TTX binding sites in skeletal muscle, but rather to study the binding characteristics of both the normal and denervated muscles of the rat so that a valid comparison between the two may be made, and hopefully, clues as to the development of the TTX resistant action potential after denervation could be provided.

METHODS AND MATERIALS

TISSUE PREPARATION

The one mammalian skeletal muscle that has been subjected to most investigations is the extensor digitorum longus (EDL) of the rat. This muscle is appropriate for denervation studies as it is easily accessible to surgical procedures. The contralateral muscle which is not operated on could serve as control.

The EDL, which is innervated by the peroneal nerve, was denervated by sectioning the sciatic nerve (the peroneal being one of its branches) at the thigh under ether anaesthesia. At various intervals following surgical removal of about 1 cm of the sciatic nerve, the denervated muscle along with its contralateral control was dissected out for experiments.

It is unfortunate that only mammalian muscles show TTX resistance after denervation. The frog skeletal muscle, which is more intensely studied, was shown not to develop such resistance after denervation (Colquhoun et al 1974).

PREPARATION OF TRITIATED TTX

Purified TTX, extracted from the ovaries of the pufferfish, was obtained from the Sankyo Co., Japan. 10mg of TTX were then sent to the ICN Corp. to be labeled by the modified Wilzbach method (Dorfman and Wilzbach 1959). The treated TTX was then subjected to purification as only 2 to 3 mg out of the original 10 mg would still be biologically active.

There are two successful procedures developed for purification of the radioactive TTX. One employs electrophoresis (Colquhoun et al 1972) and the other makes use of chromatography (Benzer and Raftery 1972). It is the latter method that was used throughout the project. A flow-chart of the procedure is provided on Fig. 3. Essentially the material is run through a Biogel P-2 column twice and finally through a Bio-Rex 70 column, which is a weakly acid cation exchanger. The desired fractions containing the active TTX were assayed by its ability to block the action potential of frog sciatic nerve. The purity of the product was determined by thin layer chromatography. Not all the crude TTX was purified at once. Usually it was divided into 5 batches so that each batch would last for 2 to 4 weeks, thus eliminating the risk of the breakdown of the purified product into undesirable impurities.

To determine the concentration of TTX in the purified fractions, a bioassay is necessary as no spectrophotometric or other conventional means is available. The frog sciatic nerve is used in the bioassay and its action potential is recorded extracellularly by a pair of platinum electrodes. The nerve is stimulated through a pair of platinum electrodes by a Grass SD 5 stimulator. By soaking the sciatic nerves in different known concentrations of non-radioactive TTX for 20 mins, a dose response curve of the size of the action potentials against TTX concentration was obtained. The unknown concentration of a ^{3}H-TTX solution was determined by the percent reduction in the size of the action potential using this dose response curve.(Fig. 4)

FLOW-CHART OF PURIFICATION STEPS

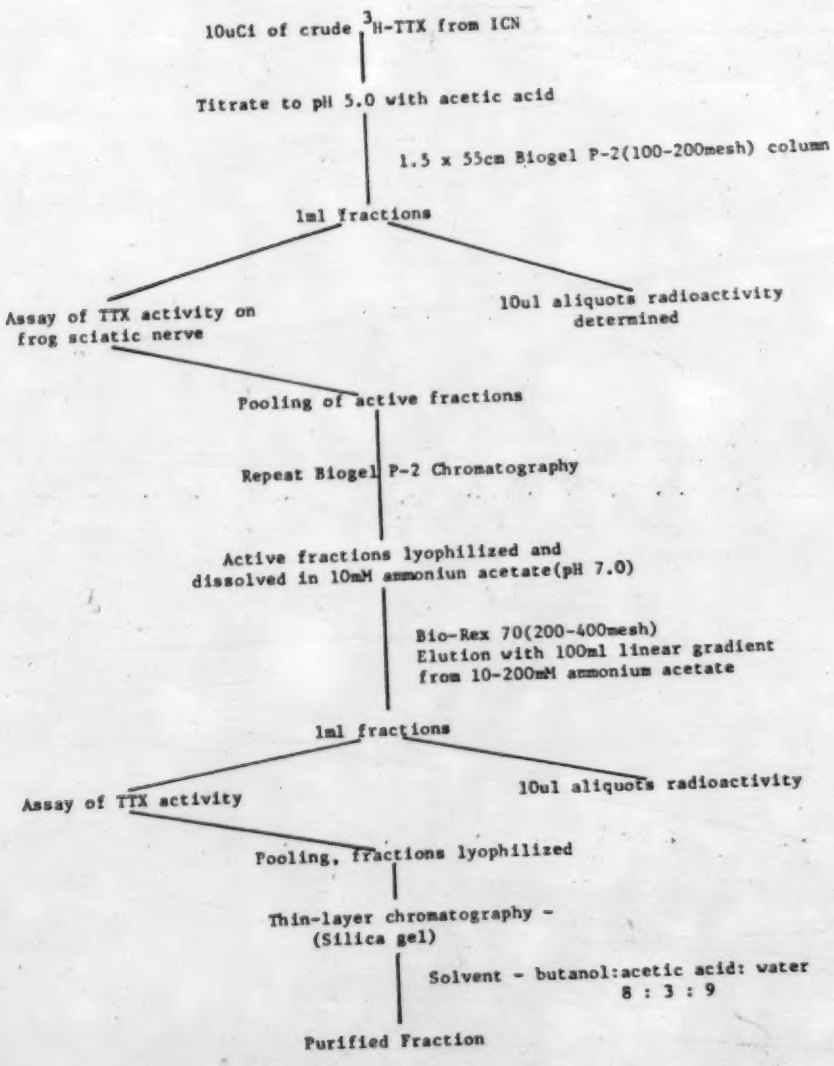
10uCi of crude ^{3}H-TTX from ICN
Titrate to pH 5.0 with acetic acid
1.5 x 55cm Biogel P-2(100-200mesh) column
1ml fractions
Assay of TTX activity on frog sciatic nerve
10ul aliquots radioactivity determined
Pooling of active fractions
Repeat Biogel P-2 Chromatography
Active fractions lyophilized and dissolved in 10mM ammoniun acetate(pH 7.0)
Bio-Rex 70(200-400mesh)
Elution with 100ml linear gradient from 10-200mM ammonium acetate
1ml fractions
Assay of TTX activity
10ul aliquots radioactivity
Pooling, fractions lyophilized
Thin-layer chromatography - (Silica gel)
Solvent - butanol:acetic acid: water 8 : 3 : 9
Purified Fraction

Fig. 3. Purification procedure of ^{3}H-TTX

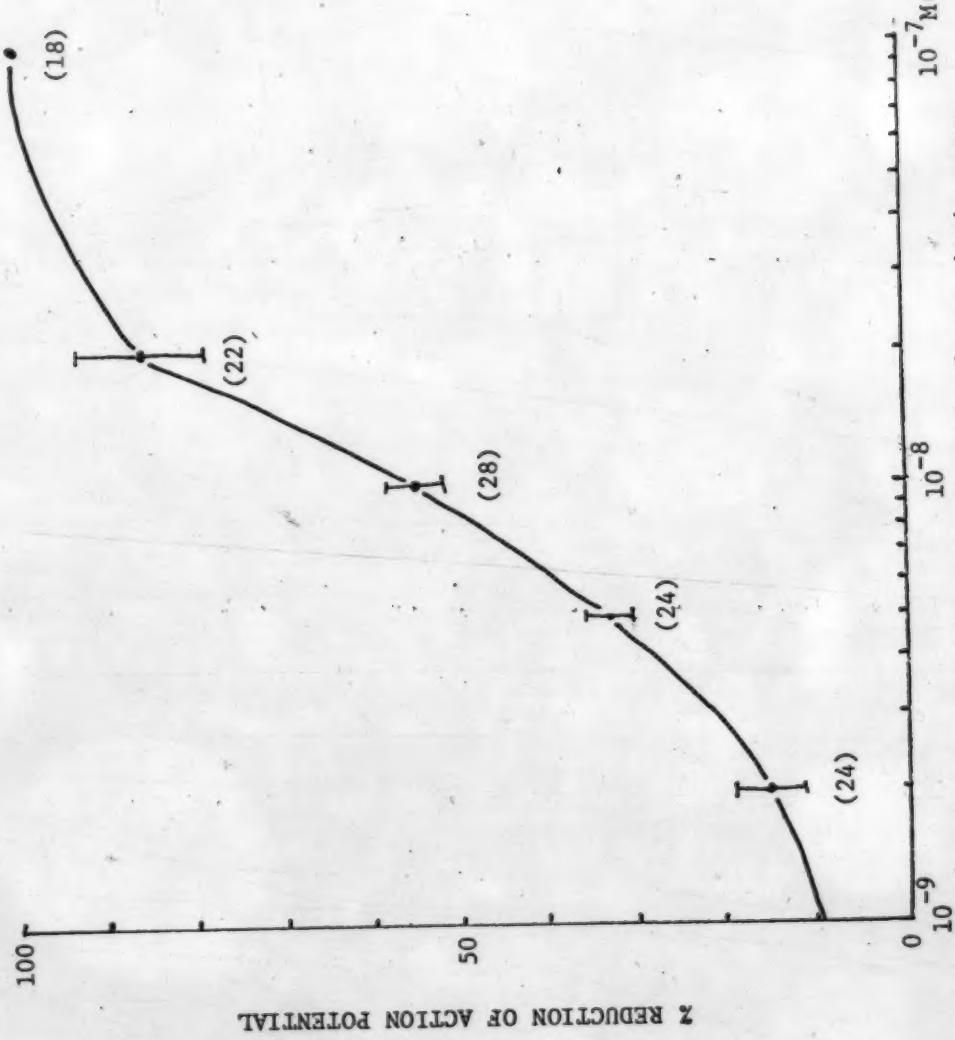

Fig. 4. Dose-response curve of TTX on frog sciatic nerve

BINDING ASSAY

Weighed rat EDLs, both normal and denervated, were removed and homogenized using a Polytron Type PT 20 homogenizer (Kinematica GmbH, Luzern-Schweiz). The crude homogenates were then spun at 650g (4,500rpm) for 15 mins with a Beckman L3-40 ultracentrifuge to remove the nuclei, contractile protein and other heavier particles. The supernatants were saved and spun into a pellet at 100,000g (35,000rpm) for 30 mins. The pellets were resuspended in physiological buffer for binding studies. All these procedures were performed at 0°C.

The rest of the procedure was done at room temperature. In a typical experiment, the final concentration of the homogenate was diluted to about 40mg of tissue per ml. The reaction mixture consisted of 1.5ml of TTX solution, 1.5ml of homogenate, and 0.5ml of Ringers solution in each tube and this was allowed to react for 15 - 20 mins before centrifugation at 100,000g for 30 mins. At the end of the run, 0.1ml of the supernatant was collected from each tube and the rest of the supernatant drained by vacuum suction and discarded. The collected supernatant fractions were counted and would be the free TTX concentrations for their respective sample. The pellets left behind were solubilized with 0.5ml of NCS (Amersham/Searle) overnight at 37°C. 10ul of acetic acid was added to each tube for neutralization before transferring the solubilized membranes to vials for counting.

The amount of free ^{3}H-TTX trapped in the pellets was estimated by a correction factor determined for each run by using 3 control tubes which contained the same amount of homogenate and made up to the same volume as those of the assay tubes. However, in these tubes, the TTX

was substituted by ^{14}C-inulin. By counting the pellets of the control tube, the volume of liquid trapped by the pellets could be calculated if from each tube, 0.1 ml of the supernatant was taken out for counting. The amount of free fluid in the pellet would be equal to (dpm pellet/ dpm of 0.1ml supernatant) x 0.1 ml. The average of three control values from each centrifugation run (together with 9 assay tubes) was used. In calculating the net amount of TTX bound per assay tube, the contribution of counts from the free TTX in the pellet was subtracted. This method eliminates much of the errors and cumbersomeness of the double-label technique.

COMPOSITION OF PHYSIOLOGICAL SOLUTIONS

Frog Ringer (Hodgkin and Horowicz 1959)

NaCl	116mM
KCl	2.5mM
$CaCl_2$	1.8mM
Na_2HPO_4	1.08mM
NaH_2PO_4	0.43mM

Rat Ringer (Liley 1956)

NaCl	135mM
KCl	5mM
$MgCl_2$	1mM
$CaCl_2$	2mM
$NaHCO_3$	15mM
Na_2HPO_4	1mM
Glucose	11mM

RADIOACTIVE COUNTING

The samples after digestion with NCS were allowed to dark-adapt for at least 48 hrs before counting. The counting efficiency for each sample was determined by the channel ratio method (Baille 1960) and the true dpm was calculated with a PDP-8 computer. The counting time for each sample was 30 mins. The Bray's Cocktail(Bray 1960) was used as fluor for all radioactive countings.

ANALYSIS OF DATA

The binding curve was analyzed by the least squares estimation of the parameters using the Patternsearch method (Colquhoun 1971). Attempts were made to find the simplest equation containing the hyperbolic saturable components of Langmuir (1918) that would fit the experimental data. (Equations 1 and 2)

$$U = b(TTX + C) + M(TTX)/(K + TTX) \qquad \text{Eqn. 1}$$

$$U = b(TTX + C) + M(TTX)/(K + TTX) + N(TTX + D)/(L + TTX + D) \qquad \text{Eqn. 2}$$

C and D are constants

The Patternsearch procedure (Colquhoun 1971, p. 263) is a program written to find the estimates of the parameters that minimize a certain function. In this case, we minimized the sum of the least squares of the relative difference $S = \Sigma((y - Y)/y)^2$ where y is the observed value and Y the calculated value. This compensates for the great difference in y at the two extreme ends. In other words, each point was weighed equally rather than dominated by the high values at the high concentrations.

The Patternsearch method starts from the initial guess of the parameters by trying steps of specific size in each variable to see whether the function S is reduced. When the function cannot be reduced further the step size is reduced and a further exploration carried out. During the estimation of the final parameters obtained here, the procedure was repeated several times so that no further improvement of the function S could be achieved.

In fitting a function, it is usually reasonable to use the simplest relationship consistent with the observations, i.e., it is desirable to find an equation that contains the smallest number of parameters, the values of which have to be estimated from the observations. (For more detailed discussion, see Colquhoun 1971, Chapter 12) In most cases, adding more parameters to the equation do not necessarily improve the estimates as shown by the size of the function S (Colquhoun et al 1972). A very important criterion to see which equation is more appropriate is their physiological relevance, which is explained in more detail in the Discussion section.

The estimation of the experimental errors were done according to Colquhoun et al (1974). In most cases, there was only one observation at each TTX concentration, therefore the sum of squares of deviations from the fitted curve, S, will be an estimate of $s^2(y)$ (Colquhoun 1971, p. 258) The approximate S.E. for the parameters were found as the square roots of the diagonal elements of the inverse of a 5 x 5 matrix $(-d^2L/dX_i dX_i)$, evaluated from the Patternsearch parameters, where $X_i (i = 1...5)$ are the parameters M, K, N, L, and b of Eqn. 2 and $L = -S/2$ is the observed log likelihood assuming Gaussian distribution.

ELECTROPHYSIOLOGICAL EXPERIMENTS

Male Wistar rats weighing 200 - 300g were used. Both the normal and the denervated EDL were taken out and bathed in normal Ringer (Liley 1956) bubbled with 95% oxygen and 5% carbon dioxide at 37°C. For temperature studies, the temperature of the organ bath was controlled by circulating water around its jacket. The muscles were mounted by strings tied to both ends of the tendons. The solution inside the bath was also bubbled with 95% oxygen and 5% carbon dioxide.

Recording Details

Essentially only the surface fibers were studied. Intracellular recordings were made with 3M KCl microelectrodes having resistance of 8 - 20 Megohms. The muscles were stimulated by a pair of external platinum electrodes with a Grass S4 stimulator through a Grass SIU-4B stimulus isolation unit. The voltage change was recorded through a WPI M4A electrometer and differentiated electronically by an op-amp circuit. The records were displayed on a Tektronix 5031 storage oscilloscpe. For TTX and imidazole studies, the muscles were allowed to soak in the drugs for at least 20 mins before recording, and 30 mins were allowed for equilibration to any change of temperature in the temperature studies.

As the denervated muscle is depolarized after denervation, it would be necessary to adjust the membrane potential to that of the innervated muscle so that a meaningful comparison of the data could be made. This is accomplished by passing a constant hyperpolarizing current through the recording microelectrode using the built-in bridge

circuit of the WPI electrometer. At least 4 secs were allowed for the fibers to adjust to the new potential to ensure complete activation of the Na channels at that particular membrane potential.

Drugs and Solutions

TTX (Sankyo Co., Japan) in 3.1 x 10^{-6}M stock Ringer solutions were kept in the refrigerator and proper dilutions were made before each set of experiments. Imidazole (Sigma) were prepared in 10^{-2}M Ringer stocks and also diluted to the desired concentrations just before each set of experiments.

RESULTS

BINDING OF TTX TO NORMAL RAT EDL

Since as little as 10^{-9}M TTX has some effect but close to 10^{-6}M is required to block the generation of action potential completely in EDL, it is within the range of 10^{-9} to 10^{-6}M TTX concentration that binding assays were done. From reports of similar experiments on nerves, it was known that TTX binds saturably to a hyperbolic component and also binds non-specifically to a linear component (Colquhoun et al 1972, Henderson et al 1973). Therefore when analysing the binding curves, attempts were made to distinguish a saturable component that might be associated with its physiological action.

As the range of TTX concentration used is over a 1000 fold, two curves were plotted to show the binding. Fig. 5 shows binding of TTX at the low concentration region and Fig. 6 is more concerned with the binding at higher concentrations. The experimental data were processed by computer for curve fitting using the Patternsearch method, which performs least squares estimation of the parameters. Using the same computer program, Colquhoun et al (1972) were able to fit their data to the function

$$U = b(TTX + C) + M(TTX)/(K + TTX) \qquad \text{Eqn. 1}$$

i.e. the uptake, U, is defined by a linear component of slope b, and a hyperbolic saturable component, which they identified as the specific physiological binding site. K is the equilibrium constant and M the binding capacity for that site.

By fitting the experimental data (from 38 observations) to Eqn. 1, the best parameter estimates were: $b = 0.0448 \times 10^{-11}$ moles/g wt; $M = 2.09 \times 10^{-11}$ moles/g wt; and K = 20nM. The values of U and the

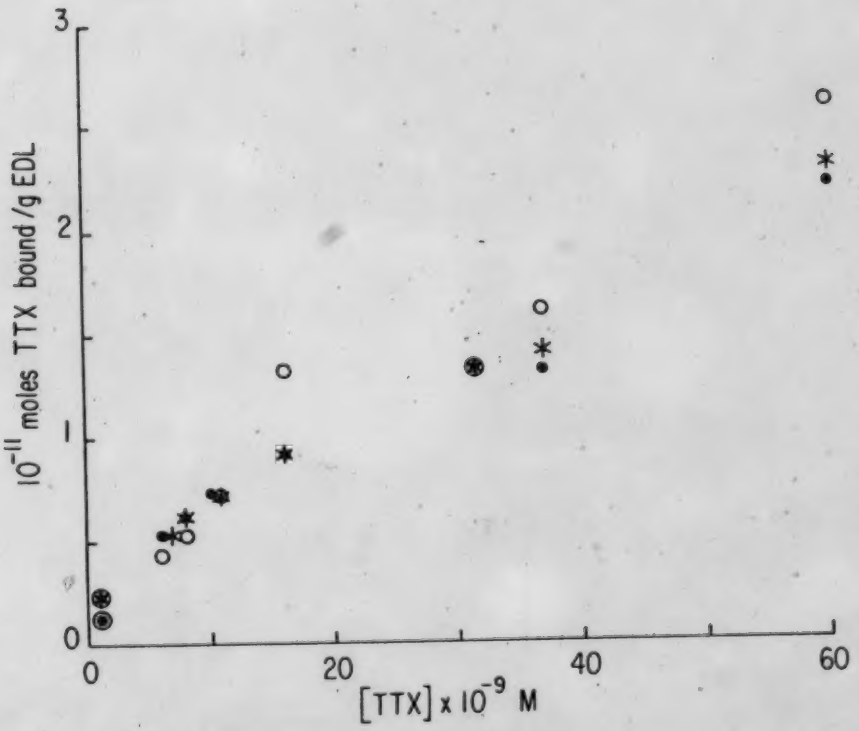

Fig. 5. Binding of TTX (low concentrations) to normal rat EDL. ○ Experimental, ✳ 1 component function from Eqn. 1, and ● 2 component function from Eqn. 2.

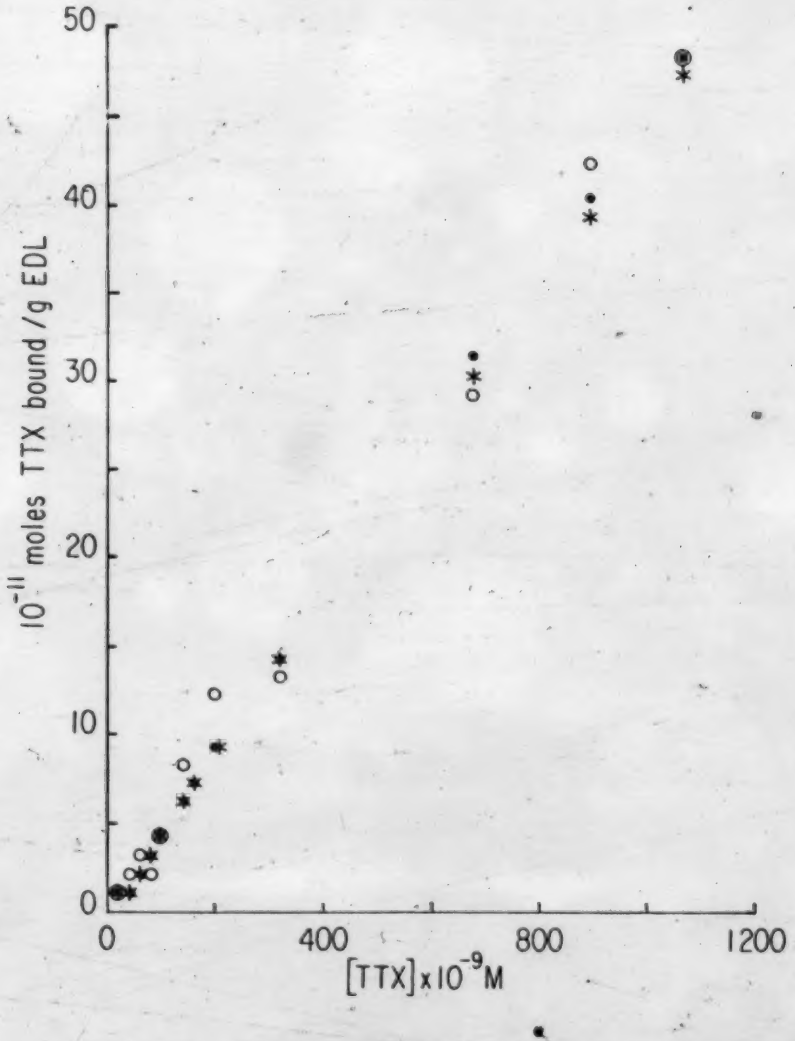

Fig. 6. Binding of TTX (high concentrations) to normal rat EDL. O Experimental, * 1 component function from Eqn. 1, and ● 2 component function from Eqn. 2.

same experimental data could be fitted equally well in terms of the minimized function S with the equation

$$U = b(TTX + C) + M(TTX)/(K + TTX) + N(TTX + D)/(L + TTX + D) \quad \text{Eqn. 2}$$

i.e. instead of one saturable component, there are two hyperbolic components. The parameters determined with Eqn. 2 were: $b = 0.0419 \times 10^{-11}$ moles/g wt, K = 3.856nM, $N = 2.939 \times 10^{-11}$ moles/g wt and L = 71nM. The reason for fitting the data with two hyperbolic components is discussed below. The ratio of the function S for Eqn. 1 to Eqn. 2 is 1.097. The binding data, along with the two theoretical curves from equations 1 and 2 were also plotted on a log-log scale for easy comparison.(Fig. 7)

BINDING OF TTX TO DENERVATED RAT EDL

The contralateral side of the control (normal) EDL was removed 4 days after denervation. The experimental procedures for binding assays of the denervated muscles were exactly the same as that on the normal control. The weight of the denervated muscle is often reduced by 2 - 8% as compared to the control. As muscles undergo active protein synthesis after denervation (Margreth et al 1966, 1972), thus altering the protein composition of the muscle, it is preferable to express the binding in terms of its weight rather than protein concentration.

A wider range of TTX concentrations was used (up to 10^{-5}M) in the binding assays for the denervated muscles as it was reported that 10^{-6}M could only partially block the action potential of the denervated muscle (Redfern and Thesleff 1972). Fitting the experi-

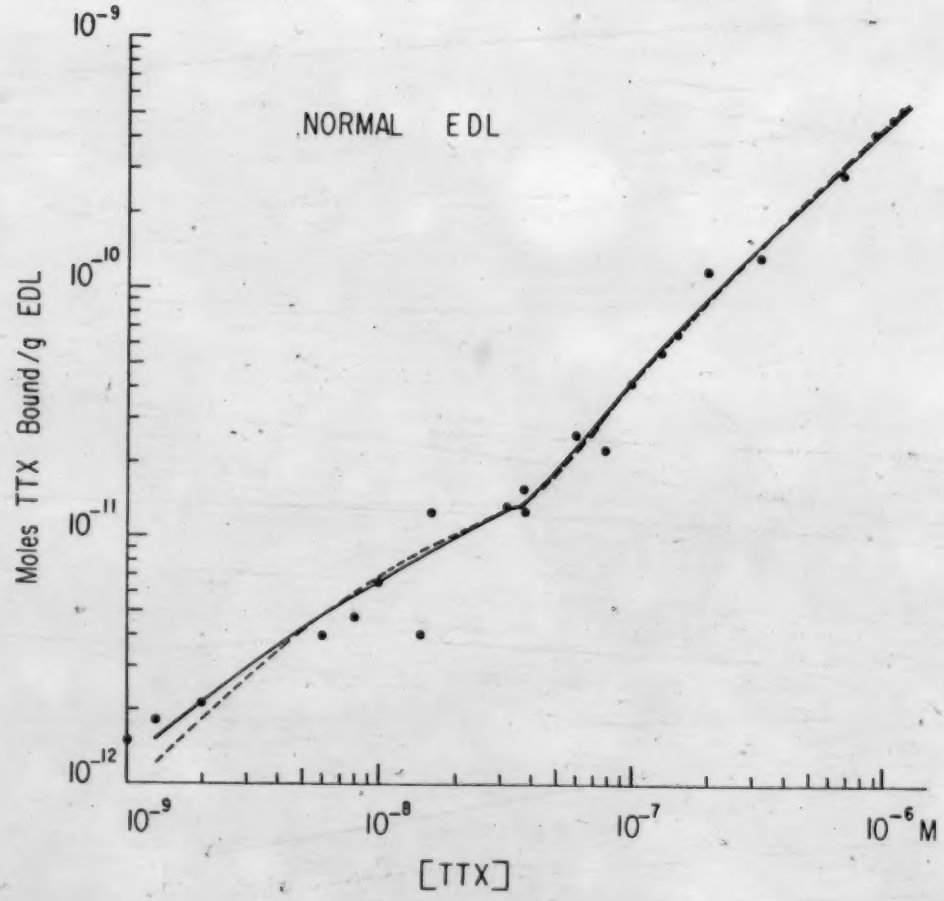

Fig. 7. Complete binding curve plotted on a log-log scale: (•) experimental points; (--) theoretical curve from Eqn. 1 with one saturable component; (—) theoretical curve with two saturable components. There is no discernible difference between the two theoretical curves.

mental data to Eqn. 1 was found not to be very satisfactory in terms of the function S. It was found that by adding a second hyperbolic component to the function as in Eqn. 2 would much improve the fit. This is evidenced by the much lower sum of least square value S - the ratio of S_1 from Eqn. 1 to S_2 from Eqn. 2 being 2.04.

The binding values against TTX concentrations were plotted in Fig. 8 for the low concentration region and in Fig. 9 for higher concentrations (2 points of higher concentrations were left out: they are 51.34×10^{-11} moles/g at 2.35×10^{-6} M and 226.58×10^{-11} moles/g at 1.17×10^{-5} M; the corresponding estimated values were 56.458 and 250.67×10^{-11} moles/g respectively). The estimated parameters for the normal and denervated EDL from both equations are summarized in Table 3. The experimental points and the theoretical curves were also plotted on a log-log scale in Fig. 10.

After denervation, the parameters of the first component M and K remain essentially unchanged, if we look only at the parameters from Eqn. 2. It is the N and L parameters of the second component that undergo the most drastic change. The N value after denervation increases by fourfold while the L value is shifted from 71nM to 538nM. Fig. 11 and Fig. 12 show the three theoretical components of the denervated EDL according to Eqn. 2.

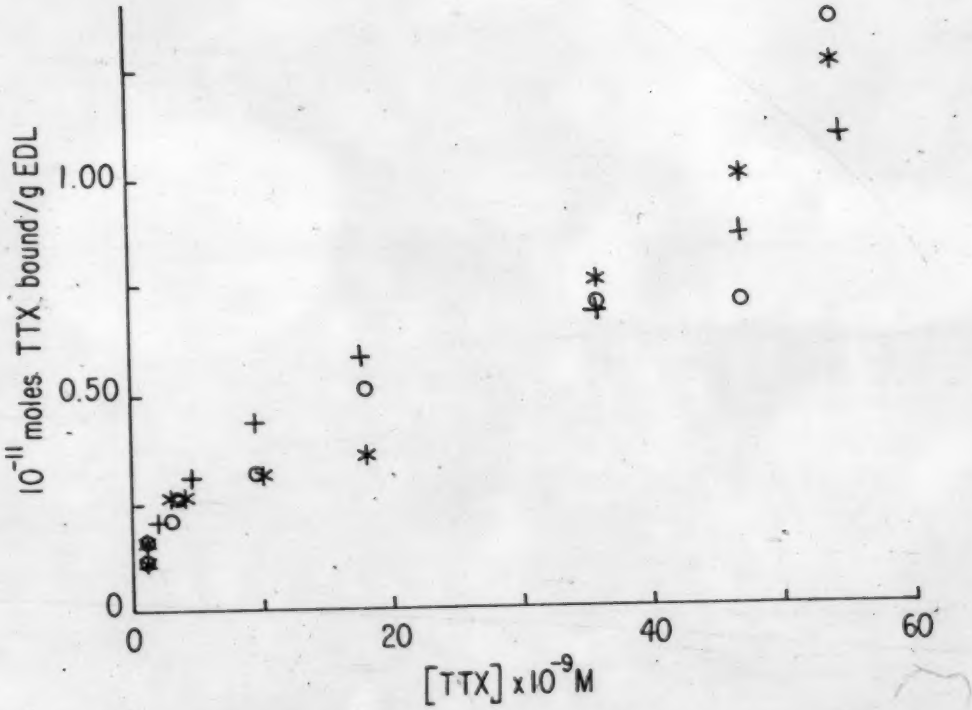

Fig. 8. Binding of TTX to denervated EDL. (o) experimental, (+) one saturable component from Eqn. 1, (*) two saturable components from Eqn. 2.

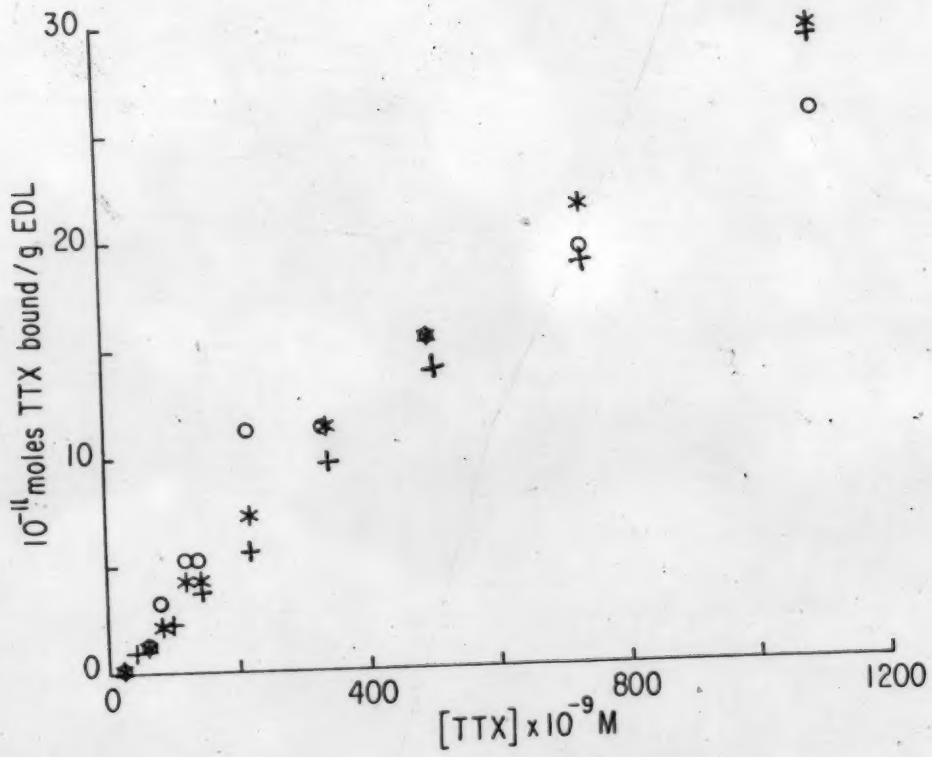

Fig. 9. Binding of TTX to denervated EDL (high conc.) (o) experimental, (+) one saturable component from Eqn. 1, (*) two saturable components from Eqn. 2.

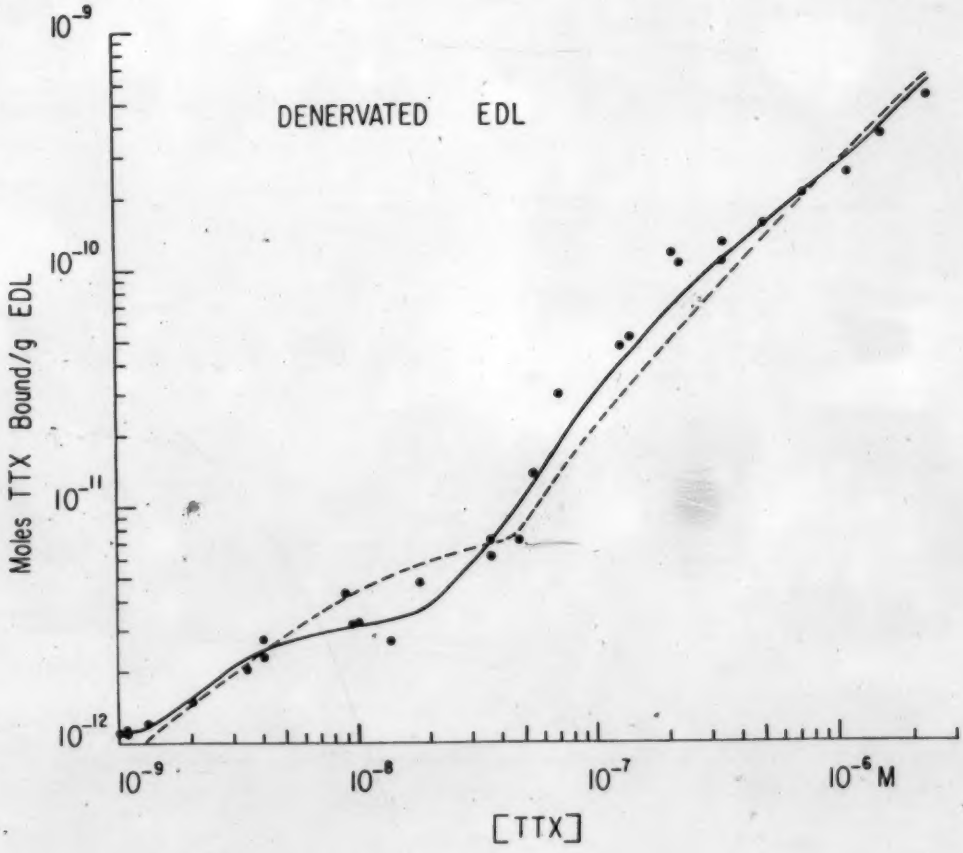

Fig. 10. Complete binding curve plotted on a log-log scale: (•) experimental points; (--) theoretical curve from Eqn. 1 with one saturable component; (—) theoretical curve with two saturable components.

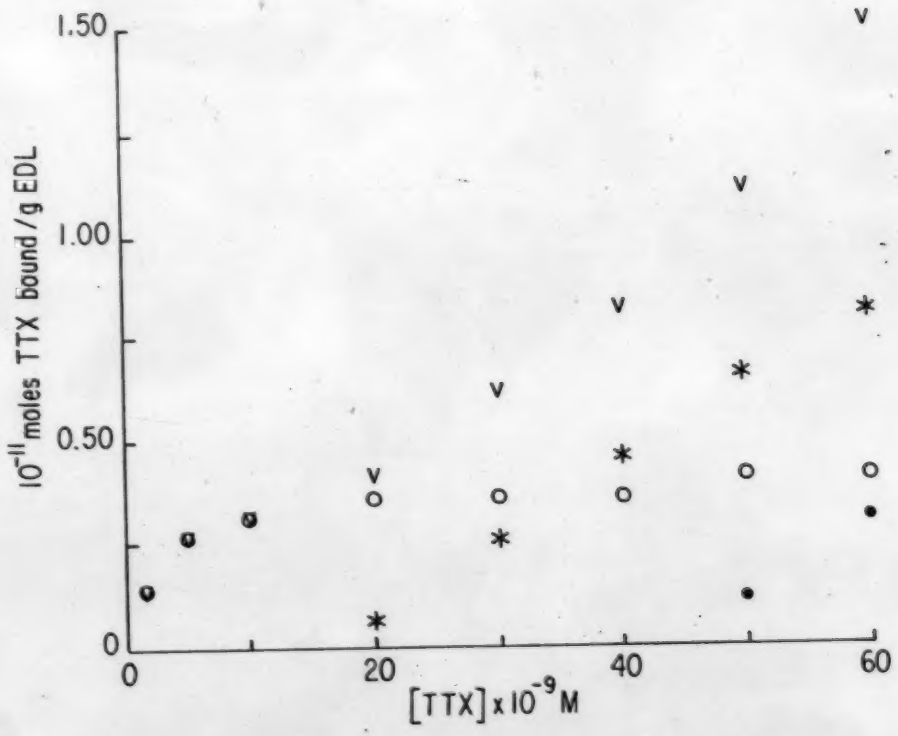

Fig. 11. The components of the theoretical function for the binding of TTX to denervated EDL from Eqn. 2.

O the first hyperbola, * the second hyperbola,

● the straight line, and V the sum of the components.

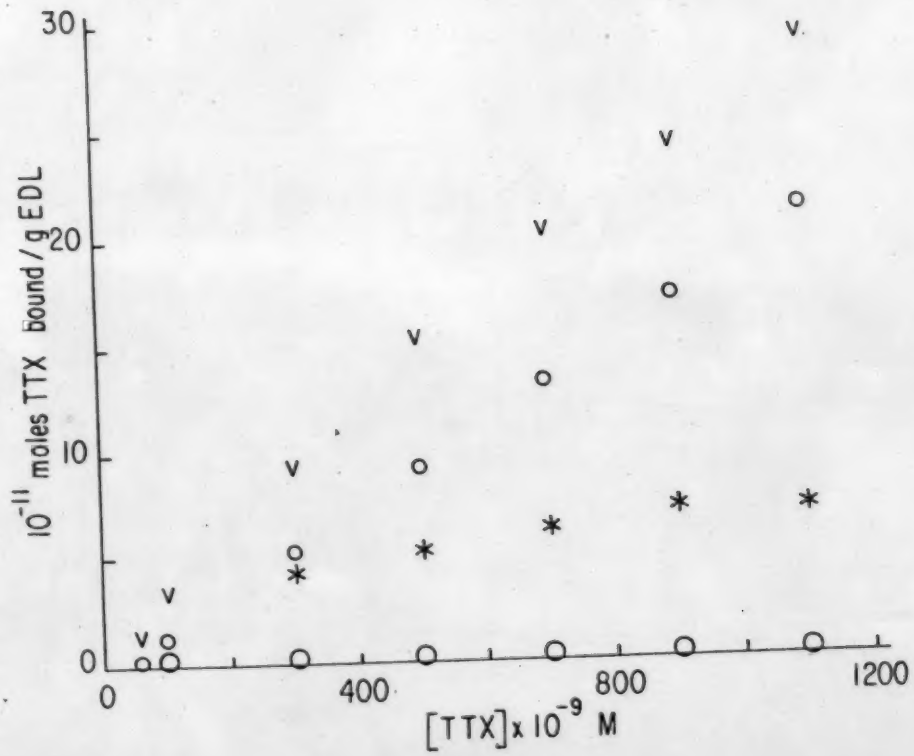

Fig. 12. The components of the theoretical function for the binding of TTX (high concentrations) to denervated rat EDL from Eqn. 2. O the first component, * the second components, and o the straight line. V is the sum of all three components.

Table 3. Estimated parameters for Eqn. 1 & 2 by Patternsearch Method

	NORMAL EDL		DENERVATED EDL	
	Eqn. 1	Eqn.2	Eqn.1	Eqn.2
b (10^{-11}M/g wt)	0.0448	0.042 ± 0.00047	0.0268	0.020 ± 0.00007
M (10^{-11}M/g wt)	2.09	0.402 ± 0.017	0.853	0.398 ± 0.018
K (nM)	20.0	3.856 ± 0.332	9.5	2.590 ± 0.231
N (10^{-11}M/g wt)		2.939 ± 0.058		11.224 ± 0.169
L (nM)		71.445 ± 7.362		538.270 ± 21.74

ELECTROPHYSIOLOGICAL EXPERIMENTS

There are two sets of parameters that fit the experimental data from the binding experiments. It was then hoped that the binding parameters of one of the sets could be correlated well with electrophysiological measurements so that a suitable model of the Na channels in the mammalian skeletal muscle could be proposed.

Effect of Temperature on the Rate of Rise of the Normal EDL

Temperature variation from 20°C to 38°C has no significant effect on the resting membrane potential. The amplitude of the action potential, however, is decreased with higher temperature and reaches a plateau at around 30°C. (Fig. 13)

Looking at the maximal rate of rise of the action potential, which measures the rate of Na entry during the action potential (Hodgkin and Katz 1949a), a very drastic transition is observed at around 32° - 33°C. (Fig. 14) From 21°C to 31°C, the Q_{10} is 1.07 and shows little temperature dependence. From 34°C to 37°C, the Q_{10} is 1.29. Thus there appears to be two different temperature dependent components.

Effect of Temperature on Denervated EDL

The resting membrane potential of rat EDL denervated 4 - 10 days were much lower than the normal resting membrane potential of 71mV. They ranged from 52mV to 60mV. By passing a constant anodal current through the recording microelectrode, the membrane potential at the

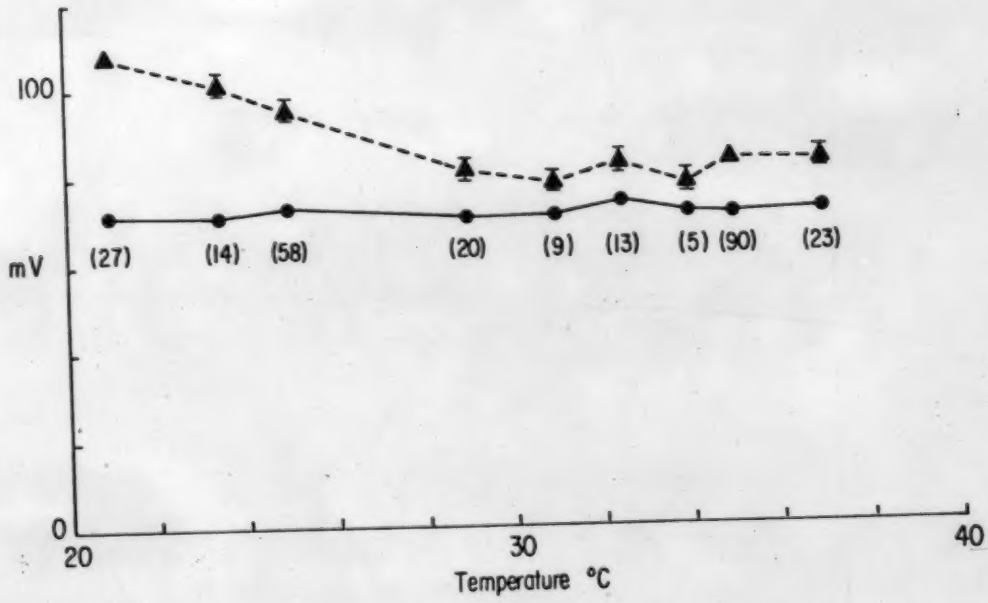

Fig. 13 . Effect of temperature on action potential and resting membrane potential of normal rat EDL. ▲ action potential, ● resting membrane potential.

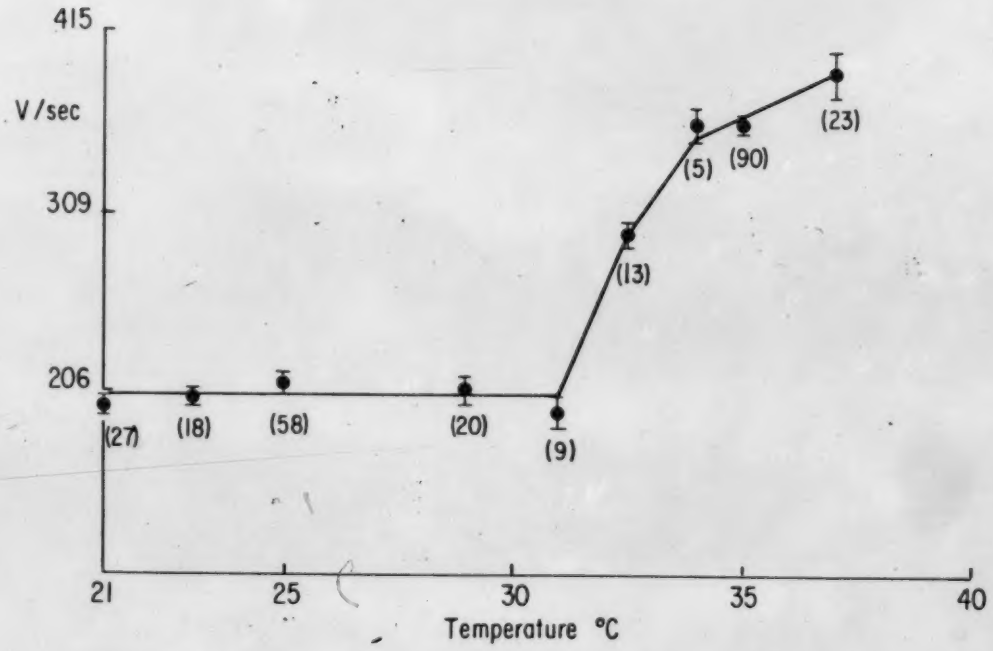

Fig. 14. The effect of temperature on the max. rate of rise of the action potential of the normal rat EDL.

site of penetration was hyperpolarized to around 71mV, the resting membrane potential of the innervated muscle. As in innervated muscle, the temperature effect on the rate of rise of the action potential was then studied.

It was found that the effect of temperature on the denervated EDL was similar to that on the normal muscle. Temperature has little effect on the rate of rise for temperatures below 30°C. There was also a sharp transition around 32° - 35°C, as in the normal muscle. In fact, it looked as if the whole temperature curve for the normal EDL was shifted downward by a factor of about 100V/sec. (Fig. 15)

Effect of TTX on Normal EDL

As the temperature affects the rate of rise quite dramatically, it would be interesting to study how the change of temperature would affect the action of TTX. Fig. 16 shows that TTX at 3.1×10^{-9}M reduces the rate of rise of the action potential at 35°C, whereas up to 7.75×10^{-9}M of TTX has little effect at 25°C. At high concentrations, the two curves of 25°C and 35°C merge and excitation is completely abolished at 3.1×10^{-7}M at any temperature. The effect of TTX on the action potential is very similar at 30°C and 25°C. (Fig. 17)

Effect of Imidazole on the Rate of Rise of the Normal EDL

Imidazole is a simple molecule with a ring structure and contians part of a guanidinium group, thus bearing some resemblance to the TTX and the STX molecule. Several imidazole derivatives have been

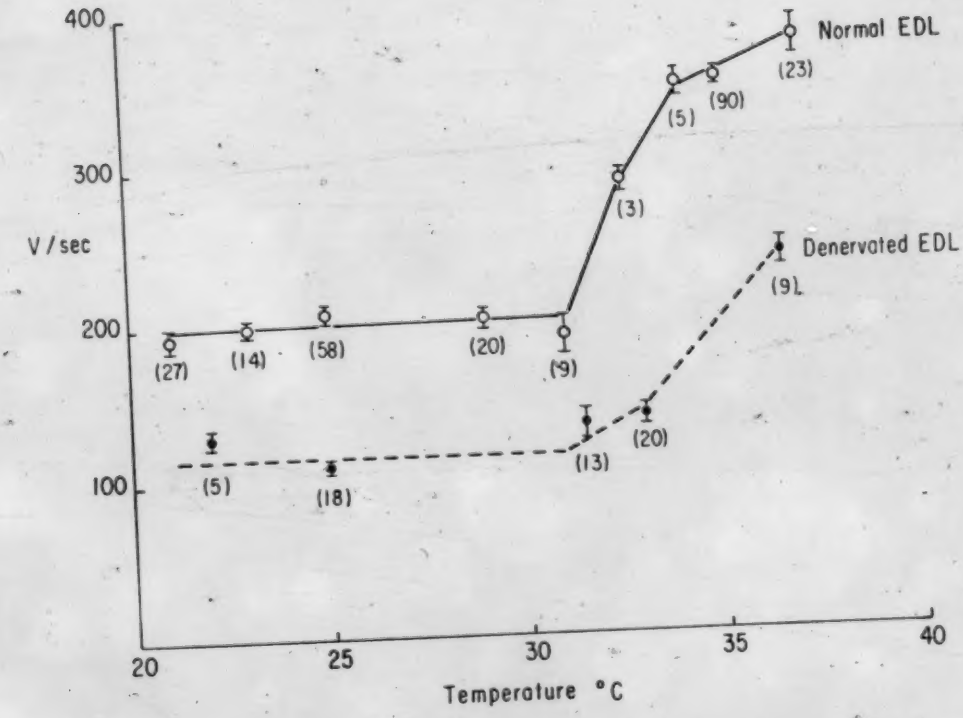

Fig. 15. Effect of Temperature on the max. rate of rise of the rat EDL.

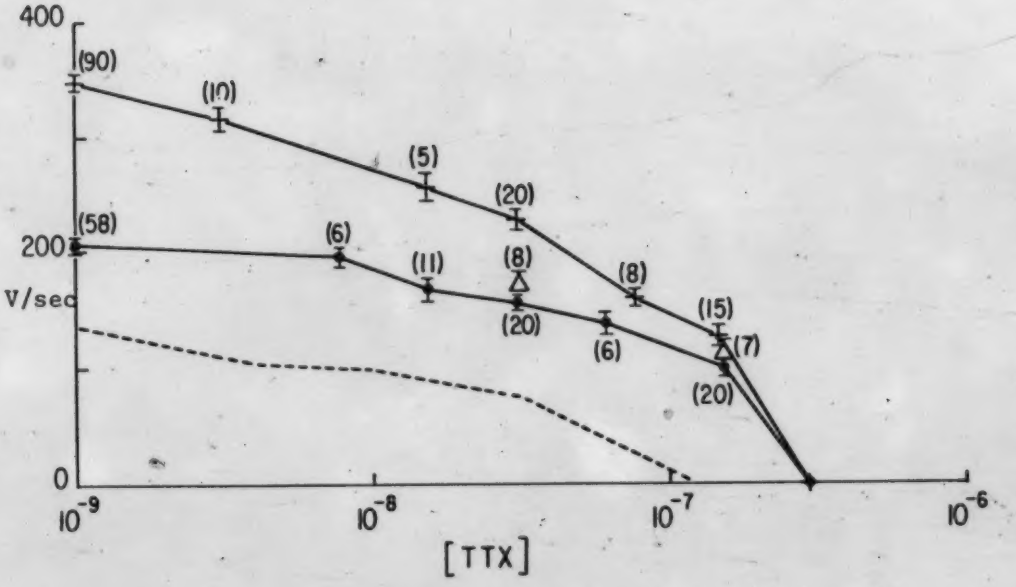

Fig. 16. Dose response curves of the effect of TTX on the max. rate of rise of the normal rat EDL. + 35 °C, Δ 29 °C, ● 25 °C. (---) is the difference between the 35 °C curve and the 25 °C curve.

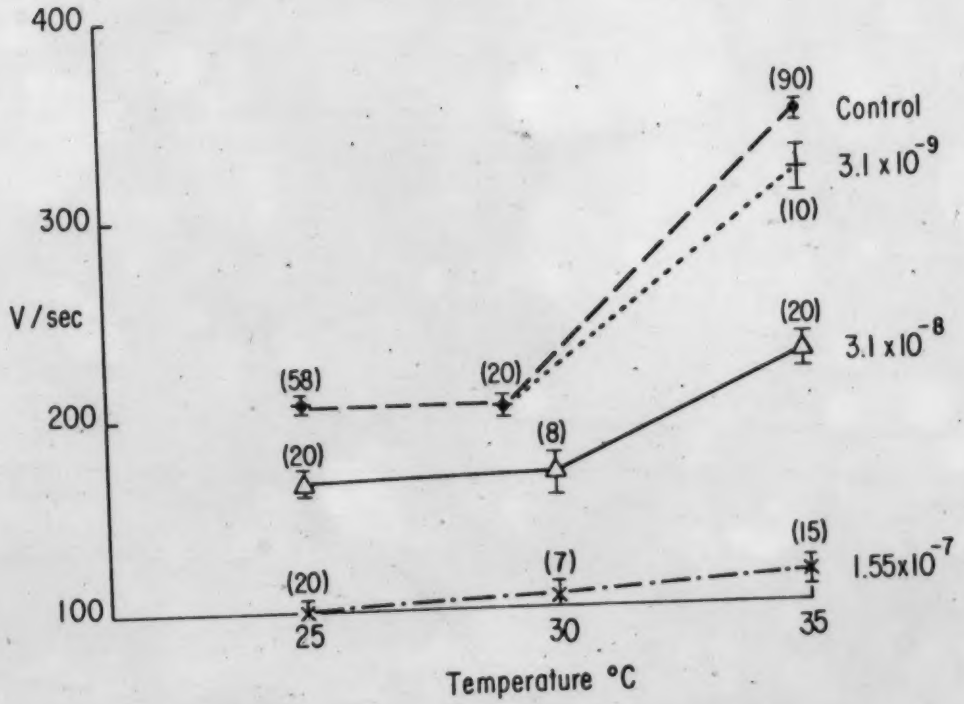

Fig. 17. The effect of temperature on TTX sensitivity.

shown to be selective against sodium conductance of nerves and frog skeletal muscles (Goldberg and Kao 1973). Therefore, the effect of imidazole on the action potential of rat skeletal muscle was investigated.

Imidazole had no effect on the resting membrane potential (Table 4), nor did it appear to have any effect on the time course of the action potential (Fig. 18). At 10^{-6} to 10^{-5}M, it reduced the rate of rise of the action potential of the normal EDL at 35°C to about 206V/sec, and higher concentration had little further effect. However, imidazole had very little effect on the rate of rise if measured at 25°C, which remained very close to the control value of about 200V/sec. (Fig. 19) The lack of effect of imidazole at 25°C is more prominent in Fig. 20 in which the action potential was shown not to alter at concentrations of imidazole ranging from 10^{-6}M to 10^{-3}M.

Effect of TTX on Imidazole Treated Normal EDL

The dose response curve of TTX in the presence of imidazole at 35°C is very similar to that done at low temperature, i.e., the sensitivity to TTX is from 10^{-8} to 10^{-6}M. (Table 5) Preliminary experiments with imidazole produced similar results with the denervated EDL.

Table 4. Effect of Imidazole on Resting Potential

Imidazole (mM)	35°C	25°C
0.005	69.11± 0.38 (9)	-
0.025	69.85 ± 0.14 (7)	-
0.05	70.87 ± 0.64 (8)	71.38 ± 0.58 (13)
0.5	71.0 ± 0.47 (14)	71.76 ± 0.74 (17)
1.0	69.84 ± 0.42 (13)	71.62 ± 0.68 (8)
2.5	70.66 ± 0.46 (18)	72.0 ± 1.0 (6)
5.0	70.2 ± 0.49 (5)	70.11 ± 0.11 (9)

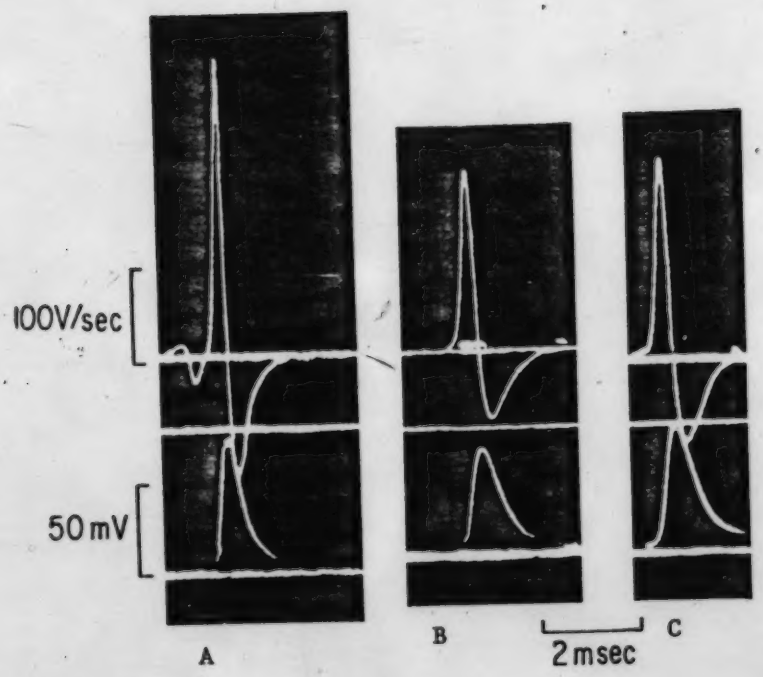

Fig 18. Intracellular recording of a typical action potential (lower trace) and its first derivative (upper trace) from an innervated rat EDL (A) at 35°C. Imidazole (10^{-4}M) reduced both the size and the max. rate of rise of the action potential (B). Addition of 7.75×10^{-9}M TTX do not change the action potential significantly (C). See text for further discussion.

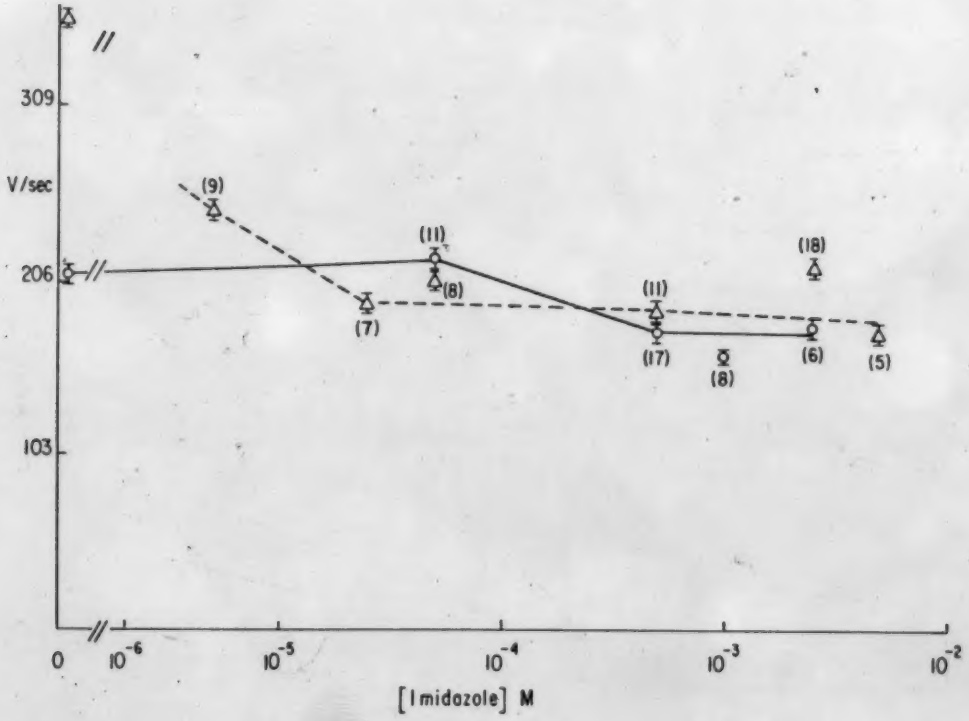

Fig. 19. The effect of imidazole on the max. rate of rise of the action potential of the normal rat EDL at (Δ) 35 °C, and (O) at 25 °C.

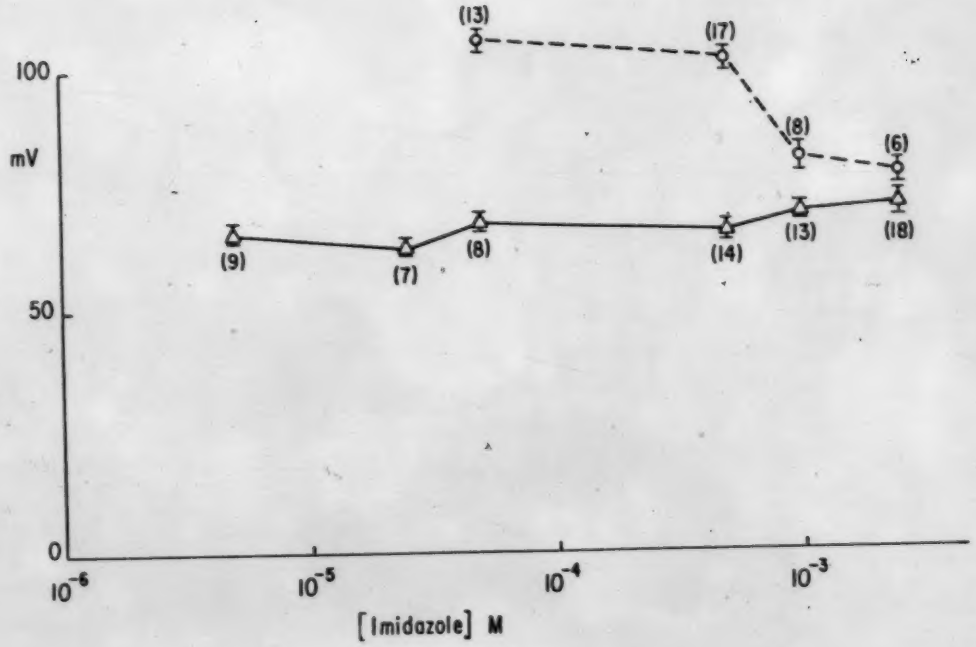

Fig. 20. The effect of imidazole on the amplitude of the action potentials at 35°C (Δ), and at 25°C (o).

Table 5. Effect of TTX on the Rate of Rise

TTX	25°C	35°C (Imidazole 10^{-4}M)
7.75×10^{-9}M	193.8 ± 11.7V/sec(6)	190.8 ± 7.0V/sec (6)
3.1×10^{-8}M	164.7 ± 5.3 (20)	163.8 ± 7.5 (17)
1.55×10^{-7}M	98.9 ± 5.2 (20)	107.2 ± 8.3 (6)

DISCUSSION

Using the Patternsearch method, it was found that the binding curves could be fitted either with one saturable component or with two saturable components. In fitting a function, it is desirable to find an equation that contains the smallest number of parameters that is at the same time relevant to its physiological function. Since the parameters are subject to errors in curve-fitting, all the possible interpretations must be exhausted and compared with physiological observations. The curve-fitting procedure with eqns. 1 and 2 suggests three most likely ways to interpret the binding data: 1) There is only one type of channels in both the normal and the denervated rat EDL. 2) There is one type of channel in the normal EDL but two types after denervation. 3) There are two types of channels in both the normal and the denervated rat EDL.

1) One Type of Channel

Using eqn. 1 to fit the binding data, it was found that the binding capacity, M, was decreased from 2.09×10^{-11} moles/g to 0.853×10^{-11} moles/g. The dissociation constant, K, also shifted from 20nM to 9.5nM. This implies that there are fewer channels in the denervated muscle, which may then explain for the reduction in the rate of rise of the action potential.

However, this interpretation with one type of Na channel appears to be inconsistent with the partial TTX resistance. It is

obvious from Table 6 where the percent occupancy of the binding sites was calculated according to the Law of Mass Action that all the channels would have been filled at concentrations close to 10^{-7}M. Electrophysiological studies, on the other hand, had shown that close to 10^{-6}M TTX was required to block the normal muscle and greater than 10^{-5}M in the case of the denervated muscle (Redfern and Thesleff 1971b).

2) One Component in Normal and Two Components after Denervation

The TTX resistance, which is not satisfactorily explained with a one channel model, could be argued as the reduction in the number of normal channels with the simultaneous formation of a new population, either through protein synthesis or by conversion of some of the normal channels after denervation.

A similar proposal has been put forward by Colquhoun et al (1974) from their study of the rat diaphragm muscle. Using the same Patternsearch method for estimating their parameters, they fitted their data to Eqn. 1 with one saturable component. These authors concluded that there is one type of channels in the normal muscle. After denervation, the number of these channels is reduced by one-half. The TTX resistance, they reasoned, is due to another population of Na channels developed after denervation.

3) Two Types of Na Channels in both the Normal and the Denervated EDL

Eqn. 2 suggests a two component model. The two components have different affinity to TTX. After denervation, there is drastic change in the component with the lower TTX affinity so that the binding

TABLE 6. % Occupancy of sites

	NORMAL		DENERVATED	
	K	M	K	M
	3.85nM	71nM	2.0nM	579.0nM
Conc. x 10^{-9}				
10	72	12.3	83	1.7
100	90	58	100	14
500	100	87.5		45.9
1000		99.3		63
3000		100		83
5000				89
10,000				94.5

capacity, L, increases almost fourfold and the affinity to TTX decreases by a factor of about 7.5.

By fitting the experimental data with the two component equation, there is some improvement in terms of the minimized function S, especially for the denervated muscle. Thus the ratio of S estimated from Eqn. 1 to that obtained with Eqn. 2 was 1.097 in the case of the normal muscle and 2.04 for the denervated muscle. Whether this improvement is statistically significant or not remains to be determined. In certain cases, adding more parameters to an equation do not necessarily improve the estimate S significantly. For example, in a binding study with the garfish olfactory nerves, it was found that the data fits a single component equation. Adding a second saturable component did not improve the fit (Colquhoun et al 1972).

Electrophysiological Experiments

From analyzing the binding data, three likely models were proposed of the Na channel system in mammalian skeletal muscle. However, due to the limitations of the technique, it was not possible to resolve the different possibilities. The physical and pharmacological properties of the channels, on the other hand, could be studied directly through electrophysiological measurements. It was hoped that the electrophysiological observations could be correlated with the binding parameters so that an appropriate model, which satisfies both the binding parameters and the electrophysiological properties, could be found. In the ensuing discussion, the electrophysiological data were interpreted broadly in the context of a one component system and a

2 component system without direct reference to the binding models.

1) One Component System

There is a temperature effect on the Na channels so that a transition occurs at around 32°C. This transition may be the consequence of a gross membrane phase change which also affects the excitation process.

The action of TTX is also affected by temperature. On the basis of electrophysiological experiments, Colquhoun and Ritchie (1972) suggested that the equilibrium dissociation constant for TTX decreases with decreasing temperature. Kinetic studies on the rate of action of TTX on the myelinated nerve fibres of Xenopus under voltage clamp conditions showed that the dissociation constant has a Q_{10} of 1.53 (Schwartz et al 1973). Therefore, if temperature were to have any effect on the action of TTX, it would mean increased sensitivity at lower temperature. Contrary to this expectation, TTX did not have any discernible effect on the rate of rise at 25°C and 30°C at concentrations below 10^{-8}M. At 35°C, 7.75×10^{-9}M caused a noticable drop in the rate of rise. This observation could not be accounted for by the direct effect of temperature on the equilibrium rate constants. In the context of a one component system, this would imply that some kind of structural or conformational change must have taken place at low temperature so that the TTX sensitivity is altered.

Imidazole has no effect on the resting membrane potential, but it reduces the rate of rise and the rate of fall of the action potential. Therefore, it is not as selective as TTX or STX although it is also

active at very low concentrations (10^{-6}M). At concentrations greater than 10^{-5}M, the rate of rise of the action potential was reduced to the level of that at low temperature. Increasing the concentration had no further effect.

In the presence of 10^{-4}M imidazole at 35°C, the effect of TTX is similar to that at low temperature in that more than 10^{-8}M is required to produce any effect. As in the case of the temperature effect, the action of imidazole could also be viewed as an effect on membrane fluidity. By this definition, imidazole does not interact with the Na channel specifically but causes a gross membrane effect.

This model with only one type of channels in both the normal and the denervated muscles necessitates that the effect of temperature on the rate of rise of the action potential be a membrane effect. Thus both the normal and the denervated muscles would be expected to be affected in the same way, i.e., lower activity of the channels at low temperature and vice versa. If the consequence of denervation was a reduction in the number of channels only, the rate of rise of the denervated muscle would be expected to be reduced by the same percentage at all temperatures. Fig. 15 shows that at low temperature, the rate of rise of the action potential of the denervated muscle was reduced by about 100V/sec, which is about 55% of that of the normal. At physiological temperature, the rate of rise is also reduced by approximately the same percentage.

2) Two Component System

The effect of temperature on the maximal rate of rise could

also be interpreted in the context of a two component system. At low temperature, only one type of channel is functional. The sharp rise in the rate of rise at around 30°C signifies the activation of a second type of channel , which is very sensitive to temperature.

In the preceding discussion, it was agreed that the action of TTX at low temperature could not be accounted for by the effect of temperature on the equilibrium rate constants. This discrepancy, on the other hand, could be explained with a two component system. The low temperature component is less sensitive to TTX in that more than 10^{-8}M is required to produce any reduction in the rate of rise. At physiological conditions, both types of channels are operating so that the observed action potential is really a sum of the two components. The difference of the dose response curves at 35°C and at 25°C yields a component that is sensitive to TTX at 10^{-9} to 10^{-7}M.

The action of imidazole could also be explained in a similar manner. As imidazole blocked only partially the action potential (reducing it to that of the low temperature level), one reasonable interpretation along this line would be that that the imidazole is selectively active on one type of channel, the more temperature sensitive component. This argument is based on the fact that many simpler chemicals, as compared to TTX and STX, that bear a ring structure and containing the guanidinium group were found to be effective against the Na current in nerves and frog skeletal muscles (Goldberg and Kao 1973). Several imidazole derivatives were reported to have this specific property.(Fig. 21) Thus it is very likely that the action of imidazole is direct on the Na channels.

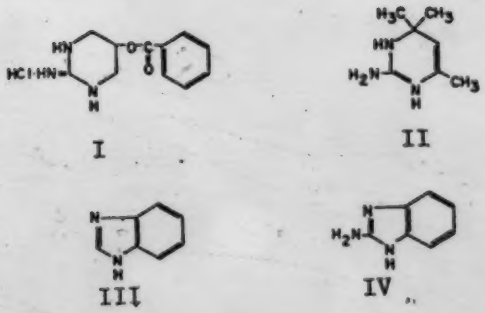

Fig. 21. Structures of
I. HM-197; II. HM-207
III. Benzimidazole and
IV. 2-aminobenzimidazole

In the presence of imidazole at 35°C, the effect of TTX is similar to that at low temperature. It thus appears that both the low temperature and imidazole affect the Na channels via a common mechanism.

If the notion that the temperature transition of the rate of rise is interpreted as the "turning on" of the second component is correct, the rate of rise of the denervated muscle then would not be expected to be reduced by the same percentage as in the previous model. Rather it would depend on how much denervation has affected the properties of each component. Fig. 15 shows that the temperature curve of the denervated muscle is depressed almost by a constant amount of 100V/sec. This would be consistant with a two component model in which only one component, the low temperature component, is drastically changed after denervation. Unfortunately, the temperature curves themselves could not differentiate between the one component model and the two component model. This is because the rate of rise just happens to be reduced both by a constant factor and by a constant percentage in this particular case. However, the work of Ward and Thesleff (1974)

shows that the two component model fits their data better under their experimental conditions when the membrane potentials were held at 100mV.

Correlation of the Binding Parameters with the Electrophysiological Observations

Although both the binding data and the electrophysiological data could be broadly interpreted with any of the three proposed models by themselves, one very important consideration in choosing the appropriate model will be the campatibility of the two sets of data acquired from two different approaches. This means that the finding with one approach has to be able to be accomodated by the other. Thus the correlation between the two sets of data allows a back check on the validity of the model. In drug-receptor interaction studies, this type of correlation is extremely important because there is no direct proof that what the binding measures is what is hoped to be the specific sites of action. In most cases, the binding is considered specific if the drug-receptor interaction demonstrates high specificity, saturability, and the affinity and kinetic rate constants of binding correlate with the biological properties of the tissue under normal physiological conditions. However, this is not always easy, and at times, not that well defined. For example, for years what was regarded as the binding of ^{3}H-noradrenaline to catecholamine receptors in intact cells and microsomes may prove not to be the case, but rather binding to a membrane catechol-binding protein which may be related to the enzyme catechol-O-methyl transferase, although the "drug-receptor" interaction

satisfies many of the criteria listed above (Cuatrecasas et al 1974). Since the parameters of a binding model prescribe a certain pharmacological and physiological responses, the validity of a model thus could be tested by how well the electrophysiological observations correlate with these prescribed parameters of the binding model.

One Component Model

The binding parameters from equation 1 prescribe a non-specific linear component and a specific binding component with a binding capacity of 2.01×10^{-11} moles/g in the normal muscle. After denervation, the binding capacity is reduced to 0.853×10^{-11} moles/g. If the properties of the Na channels were to remain unchanged after denervation, one would expect the maximal rate of rise of the denervated muscle to be reduced by approximately 50%. This was found to be the case in the present study.

Although this model is simple and thus most worthy of consideration, the fact that it cannot provide proper explanation for other physiological observations renders it a very weak working model. The problems with this model can be summarized as follows:

1) The TTX sensitivity range calculated from the dissociation constants do not match the dose response curves from electrophysiological studies, i.e., using the Law of Mass Action, all the sites would have been occupied at 10^{-7}M, but actually, 10^{-6}M is required to block the action potential of the normal muscle and greater than 10^{-5}M is required for the denervated muscle.

2) The reduced sensitivity to TTX at low temperature from the electrophysiological studies was contradictory to the expected outcome from the effect of temperature on the rate constants alone, i.e., rather than a decreased sensitivity to TTX at low temperature, it would be expected to be more sensitive to TTX.

3) Although the reduction in the number of channels may explain for the reduction in the maximal rate of rise after denervation, the partial TTX resistance remains to be accounted for. According to the binding model using one component, the action potential would have been eliminated with 10^{-7}M TTX before and after denervation.

4) Assuming that the temperature transition is a membrane phase change, the percentage reduction of the maximal rate of rise of the denervated muscle would be expected to be the same at all temperatures. Although this appears to be true in the present study when the measurements were taken at the resting membrane potential of 71mV, it is remarkably different if the effect of temperature were studied at a membrane potential of 100mV (Ward and Thesleff 1974). The percentage reduction is much lower at higher temperatures.

One Component in Normal Muscle and Two Components in Denervated Muscle

To accomodate for the TTX resistance, a two component model for the denervated muscle offers the necessary flexibility. Whereas the least square function, S, is much smaller from the two component equation for the denervated muscle, it is not too much different with the normal muscle. Thus by using the parameters of Eqn. 1 for the normal muscle and those of Eqn. 2 for the denervated muscle (see the

following section for the discussion of the two component system), the TTX resistance phenomenon could then be accomodated. However, as in the previous model, the parameters of the one component model defining the normal muscle also suffer from 1) the TTX sensitivity range is broader than expected from calculation from the equilibrium dissociation constant, and 2) the need to explain the reduced sensitivity to TTX at low temperature for the normal muscle.

Two Component Model

The binding parameters from this model derived from Eqn. 2 are more compatible with the electrophysiological observations. Accord- to the binding model, there are two types of binding sites. The TTX sensitivity range calculated from the equilibrium dissociation constants of 3.85nM and 71nM are 10^{-9} to 10^{-7}M and 10^{-8} to 10^{-6}M respectively for the normal muscle, and for the denervated muscle, the equilibrium dissociation constants of 2.59nM and 538nM corresponds to 10^{-9} to 10^{-7}M and 10^{-7} to 10^{-5}M respectively.

In this model, the observed transition in the maximal rate of rise of the action potential at 30°C is taken as the "turning on" of a second component. The TTX sensitivity at temperatures below 30°C was 10^{-8} to 10^{-6}M for the normal muscle. At normal physiological temperature, when both types of channels are operative, the sensitivity range was 10^{-9} to 10^{-6}M. This would correspond to the sum of a 10^{-9} to 10^{-7}M component and a 10^{-8} to 10^{-6}M component. By this interpretation, the discrepancy between the expected influence of temperature on the equilibrium dissociation constants and the decreased sensitivity to TTX at

low temperature could then be resolved.

In support of this interpretation is the effect of imidazole on the maximal rate of rise. Imidazole reduces the rate of rise to the same level as that at low temperature. The TTX dose response curve at 35°C in the presence of imidazole is also 10^{-8} to 10^{-6}M, as that of low temperature. As imidazole resembles that part of the TTX and STX molecules which is thought to be essential for their Na current blocking ability and that several imidazole derivatives had been shown to be specific against the sodium conductance, that the action of imidazole is direct on the channels themselves rather than on the membrane fluidity is more likely. The reason that imidazole, unlike TTX and STX, is selective only against one type of channels could be interpreted as the following. Since TTX and STX are molecules specially engineered by nature to block Na channels, it would not be unreasonable to propose that other parts of the molecules, other than the guanidinium group and the ring structure, could impart Na blocking ability, especially on channels that are different from the conventional ones, as in the case of mammalian skeletal muscle.

Nevertheless, in order to account for the reduction in the rate of rise of the action potential after denervation, this interpretation requires the assumption that the affinity of the channels to TTX reflects the general capability of the channels as the coordination sites for the current carrying ion. This assumption is justified by the work of Henderson et al (1974) on nerve membranes where they showed that various cations, including Na, compete with TTX for its binding sites. These authors suggested that these binding sites are the principal coordination sites for cations as they pass through the membrane

during an action potential.

With this assumption, the reduced rate of rise after denervation could then be interpreted as the consequence of reduced affinity to Na ions of the low affinity component, i.e., the dissociation constant, L, of the second component was shifted from 71nM in the normal muscle to 538nM in the denervated muscle. Therefore, despite an apparent increase in the number of these channels (the binding capacity, N, increased from 2.9×10^{-11} moles/g to 11.2×10^{-11} moles/g). the overall contribution from this component would still be reduced by a factor of two. The high affinity channel, on the other hand, remained essentially the same after denervation.

A Comparative Review of the Na Channels of other Excitable Membranes

With the help of the voltage clamp technique, perhaps the best studies on the Na channels in excitable membranes were done on non-mammalian systems, especially on nerve preparations. Kinetics studies had shown that TTX interact with the channels on a one-to-one basis (Cuervo and Adelman 1970). The activation energies for the rate of association and the rate of dissociation were found to be 9.8kcal/mole and 20.5kcal/mole respectively for the myelinated nerve fibers of Xenopus (Schwartz et al 1973). Based on voltage clamp analysis, the number of Na channels in the squid giant axon was estimated to be $480/um^2$ (Keynes and Rojas 1974), which is in close agreement with the value of $553/um^2$ obtained from TTX binding studies (Levinson and Meves 1975). The conductance of a single Na channel has been estimated to be 2.5×10^{-12}mho in the squid giant axon (Keynes and Rojas 1974). For all the nerve preparations studied, only 10^{-7}M TTX would be required to block the action potential, the only exceptions being those from the Californian newt, Taricha, which also produces TTX in its eggs, and that of the pufferfish (Kao and Fuhrman 1967, Kidokoro et al 1974).

The effect of temperature on the propagation of the action potential appears to be highly influenced by the environmental factor. Thus propagation of impulse fails in isolated mammalian or tropical frog's nerves when cooled to a temperature at which the response of the squid giant axon reaches its maximum amplitude (Gasser 1931). The rate of rise of the action potential of the squid giant axon has a Q_{10} of about 1.3 and the Na permeability measured under voltage clamp conditions also has the same temperature coefficient (Table 7).

Table 7. Effect of Temperature on the Rate of Rise & Rate of Fall

Tissue	Rate of Rise	Rate of Fall	Reference
Rat EDL (34 - 37°C)	1.29	1.53	This study
Myelinated Nerves of Xenopus (Voltage clamp)	1.30 (P_{Na})	1.20 (P_K)	Frankenhaeuser & Moore (1963)
Squid Giant Axon) (20 - 30°C)	1.29 - 1.48	1.9 - 1.95	Hodgkin & Katz (1949b)

The situation is more complex in the skeletal muscle because of the tubular system, which is very likely to be capable of generating action potentials (Constantin 1970, Adrian and Peachey 1973). The only muscle system that has been studied more extensively in this area is that of the frog skeletal muscle. The conductance of a single channel in the frog skeletal muscle has been estimated to be 10^{-12}mho (Adrian et al 1970, Idelfonse and Roy 1972 , Almers and Levinson 1975). As in most other nerve preparations, the action potential of frog skeletal muscle could be blocked by less than 10^{-7}M TTX (Idelfonse and Roy 1972, Almers and Levinson 1975).

The mammalian skeletal muscle differs from the frog skeletal muscle and the nerves in three major respects: 1) 10^{-6}M TTX is required to block the action potential although it is also sensitive, as in the other excitable systems, at concentration as low as 10^{-9}M. 2) After denervation, the mammalian skeletal muscle becomes resistant to TTX so that more than 10^{-5}M TTX is required to drastically reduce the rate of rise of the action potential. 3) Temperature causes a transition on the Na permeability at around 32^{o}C. At low temperatures, the rate of rise of the rat EDL was not very sensitive to temperature change (Q_{10}= 1.07). At physiological temperature, the rate of rise has a Q_{10} of 1.29.

Obviously, the structure of the excitation system of the mammalian skeletal muscle must be different from that of the nerves and the frog skeletal muscle. Based on their study of the rat diaphram muscle, Colquhoun et al (1974) proposed that there is one type of channel in the innervated muscle. After denervation, these channels were reduced to half of their original number, and that the TTX resis-

tance could be due to another population of channels that are not sensitive to TTX. They also estimated the conductance of a single channel to be 10^{-10}mho. However, it is known that the denervated muscle is not completely resistant to TTX. With 10^{-5}M TTX and at a membrane potential of 100mV, the rate of rise of the denervated muscle is only 10% of that of control (Harris and Thesleff 1971). Their estimate of the conductance of a single channel also appears to be too high for an ion selective channel. Even a rather non-selective ionophore like Gramacidin A has a lower value of about 10^{-11}mho (Hladky and Haydon 1972). Another problem with their study might arise from the fact that they only investigated the effect of TTX at relatively low concentrations. The highest concentration they tested was 5×10^{-7}M even though it was known that 10^{-6}M would be required to block the action potential of the normal muscle.

The present study looked into some possible models from the binding studies and electrophysiological experiments. Unfortunately, there is no conclusive evidence that any of the models investigated is correct. However, it does appear that a two component model may be more convenient in interpretating many of the physiological observations of the rat EDL.

There are supporting evidences from other excitable systems that there may be two types of channels. Some very interesting studies in this area were done on chick embryonic heart cells. Ventricular cells of young (2 to 3 days old) chick hearts were found to have small amplitude action potentials and also low maximal rate of rise. This action potential is insensitive to TTX (2.7×10^{-6}M) but Na dependent. Anodal shock excitation does not increase the rate of rise. These

observations led Sperelakis and Shigenobu to postulate the presence of a slow Na channel system in these cells (Shigenobu and Sperelakis 1971, 1972). Fast Na channels, which are sensitive to TTX, first appear on about day 5, and increase in number during development whereas that of the slow channels decreased. The slow channels have low inactivation potentials (20mV) and are not blocked by Mn nor abolished by low Ca. After day 8, the major inward current is carried by the fast channels as evidenced by the fast rate of rise (120V/sec as compared to 20V/sec before). This channel is blocked by TTX. It can also admit Li while the slow channels cannot (Sperelakis 1972).

Hearts removed from 2 - 7 day embryos and put into organ culture retain the slow channels and do not gain any fast channels (Sperelakis and Shigenobu 1974). This observation suggests that the young cell may not be programmed genetically, i.e., further development of its membrane properties must rely on some external influence. The appearance of the fast Na channels was found to coincide with the development of innervation. Rat embryonic hearts were found not to be sensitive to TTX until day 12, about the time of innervation (Gargouil and Bernard 1971). Further evidence that this might be the case came from studies of mature hearts where the membrane properties were fully developed. Mature intact hearts in organ culture do not lose the fast channels, however, when these hearts were minced and placed into monolayer cell culture, they regain the slow channels and lose the TTX sensitivity (McLean and Sperelakis 1974).

From the chick heart studies, it seems that there are two types of Na channels, which are functionally and pharmacologically different, and that the development and expression of these channels

are dependent upon innervation. A very similar mode of development of membrane excitability occurs in the rat skeletal muscle.

The L_6 line of myoblasts from rat (Yaffe 1968) have been in continuous culture for several years. The development of excitability from this clonal rat skeletal muscle cell line has been reported by several groups, and they all have similar findings. At the myoblast stage, a regenerative mechanism is present and is Na dependent. Action potentials overshoot zero potential only in the myotubes. However, in both cases, action potential could only be generated by anodal break excitation. Some of the action potentials in the myotubes are fast spikes and some are composed of two components. an initial fast spike followed by a hump or in some cases, a very distinct second peak. The fast spike is Na dependent and its overshoot decreases with decreasing external Na concentration. The second component could be evoked in Na free solution if the external Ca concentration is increased to 10mM. $LaCl_3$ could eliminate this second component leaving the initial component intact (Kidokoro 1975b). With depolarizing current, only the slow action potential was evoked as the fast component was completely inactivated (Kidokoro 1975b).

In cross-striated fibers, slow regenerative action potentials, similar to those found in the myotube were seen using $BaCl_2$ as a suppressor of the delayed rectification which curtails the hump in normal saline(Kidokoro 1973). It is noteworthy that while the fast component is TTX sensitive, the slow component is insensitive or only reduced in amplitude at 10^{-5}M TTX (Kidokoro 1973, Land et al 1973).

Foetal rat diaphragm muscles are also capable of generating action potentials in the presence of 10^{-6}M TTX and the number of fibers

capable of doing this decrease with age. Thus about 10 days post partum, all muscles could be blocked by TTX (Harris and Marshall 1973). It is also more than coincidental that the time at which all the muscle fibers are susceptible to TTX action corresponds to the time at which the normal adult pattern of endplate innervation is established (Redfern 1970). An even more interesting finding on foetal rat skeletal muscle was that while there exist some areas of the membrane which are highly sensitive to TTX (10^{-8}M), there are areas which are very resistant and require 10^{-5}M to abolish the action potentials (Vyskocil 1974).

As for the case of the denervated skeletal muscle, where it was found that 10^{-5}M TTX could not block the action potential completely, one would wonder if a true second component which is more resistant to TTX really exists. In other words, the loss of neural control could very well produce reversion of some membrane properties of the muscle to its more embryonic state. This is exemplified by the chick heart studies. The close similarity of action potential sensitivity to TTX between the denervated muscle and the foetal muscle in that both are partially resistant to TTX should be well taken into consideration. That some fundamental change in the membrane properties after denervation is also suggested by the fact that actinomycin D could prevent all of the denervation changes such as ACh supersensitivity and TTX resistance (Grampp et al 1972).

Even in the squid giant axon, two type of TTX sensitive channels have been reported (Meves and Vogel 1973). Internally perfused with CsF and placed in a Na-free solution of 100mM $CaCl_2$, the squid giant axon could produce inward currents which are blocked by TTX. This current is carried by Ca in the absence of Na, although the

current would be much larger in the presence of Na, the permeability ratio of Ca to Na being 1/10. The time course of this inward current, which is less sensitive to inactivation than the usual fast component, is different from that of the normal current.

However, it must be emphasized that the appearance of this second TTX sensitive current is a very marginal event occurring only under very special experimental conditions. The main point of this example is that multiple forms of a functional unit may be an universal phenomenon governed by some genetical or developmental factors. Therefore, the existence of two populations of Na channels in the mammalian skeletal muscle is certainly possible. It is very likely that after denervation, new channels are formed, which may be genetically identical to those of the second component of the normal muscle, but which are changed structurally and physiologically due to the drastic alteration of the membrane structure as a result of large protein and lipid turnovers (Graff et al 1965a, b, c).

There are indications that the Na channels of mammlian skeletal muscle do undergo qualitative changes after denervation. Batrachotoxin (BTX) is a very specific agent that causes depolarization of excitable membranes by opening up the Na channels (Albuquerque et al 1971). As little as 1.5×10^{-7}M BTX is required to depolarize the innervated or denervated rat EDL completely. In normal rat EDL, when 3×10^{-6}M TTX is used in combination with BTX, no membrane depolarization is observed. However, in denervated muscles, TTX's ability to antagonize the effects of BTX is correlated with the time after denervation in that TTX could partially restore the resting membrane potential in the first few days, and by day 10, TTX would be totally ineffect-

ive in preventing depolarization caused by BTX. As BTX presumably acts on a different site of the Na channel (Albuquerque et al 1975), this observation may indicate that while the BTX sites may not be affected by denervation, the TTX sites are certainly altered.

Conclusion

From the above discussion, it is true to say that no definite conclusion about an appropriate model could be drawn at this stage of study. Nevertheless, the two component model seems the most attractive in the interpretation of the physiological data. It is much easier to interpret the TTX resistance in denervated muscle in terms of a two component system. The correlation between the binding parameters and the electrophysiological data is also better in this case. Therefore, the possible existence of a two component system in mammalian skeletal muscle will have to be taken into consideration in future experimentation.

There are two possible approaches to test whether there exists a two component system in the mammalian skeletal muscle. If the two types of channels were not exactly the same, then one would expect different physical characteristics between the two. If the difference were large enough, it would not be hard to separate the two. The other way to distinguish the two components would be pharmacological; one would require an agent which could selectively act on one specific component and have little or no effect on the other.

Physically, not too much information about the Na channel is known. However, it is known that the Na channel of nerves has an an-

ionic group of pK_a 5.2 (Hille 1971, Woodhull 1973). Since the binding characteristics of one of the components in muscle appears to be very similar to that in nerves, it would not be inconceivable that this component also possesses a strong negative group. As hydrogen ion could block this type of channel (Woodhull 1973), a titration of the muscle would probably yield valuable information if the second component does not have a similar group.

It has long been worked out that ionic currents are functions of temperature and voltage. It would imply that the parameters defining these currents would be different for different types of channels. From the work done on chick embryo heart, myotubes of skeletal muscle and the internal perfused squid giant axon, in all cases where a second component was found, this second component had a lower inactivation voltage than the fast channel. Therefore, the effect of temperature, which had been investigated to some extent in the present study, and the degree of inactivation at various membrane potentials may help to resolve the two components in the rat skeletal muscle.

Pharmacologically, the ideal case would be to have some drugs that are selective for one type of channel only. The remaining channel should then exhibit TTX sensitivity as one of the components in the binding studies. In this study, the effect of imidazole had been investigated because of its structural similarity to TTX. The results indicated that the effect of imidazole may be directly on the channels. However, further experimentation need to be done to establish the specficity of its action.

Carbodiimides, which had been used to alkylate the ACh receptors by reacting with its carboxyl group (Stuesses and Katz 1973), as

well as those at the Na channels (Shrager and Profera 1973), may also be useful. However, the conditions for the experiment are very drastic.

Above were listed some of the possibilities that might help to test and differentiate the two channels. It has to be emphasized that very little basic study on the electrophysiology of the mammalian skeletal muscles has been done. The frog skeletal muscle, which is well studied in many of its membrane properties, does not develop TTX resistance after denervation (Colquhoun et al 1974).

Recently, the skeletal muscles of mice with motor-end-plate disease and ventral horn cell disease (Wobbler) have been shown to resemble the denervated skeletal muscle in many ways. They have low membrane potentials, slow action potentials, and the action potentials are resistant to TTX (Harris and Ward 1974). Therefore, the study of the denervated muscle may also contribute to the understanding of the nature of some neuromuscular diseases.

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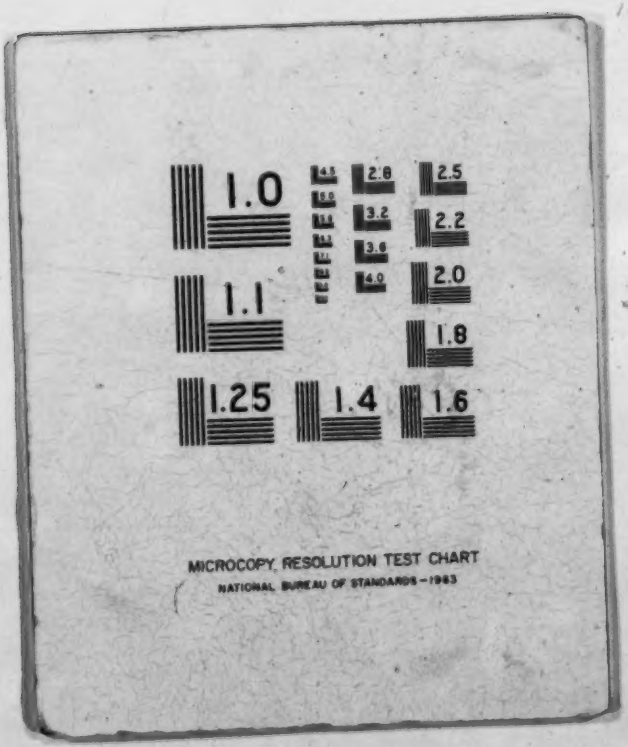

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